Chapter 3. Role of Natural Killer Cell in the Pathogenesis of Nasal Polyps and Chronic Sinusitis

3.1 Biology of Natural Killer Cells

3.1.1 Lymphocytes in Innate and Adaptive Immunity

Both specific and nonspecific immunity play important roles in protecting the host against microorganism infection. The central role of lymphocytes in adaptive immunity was discussed in chapter 1. The NK cell (natural killer cell) is an important cell in innate immunity. CD4+ and CD8+ T cells, B cells and NK cells are all differentiated from pluripotent stem cells in bone marrow under the influence of varieties of soluble factors. The proportion of T cells, B cells and NK cells in peripheral blood lymphocytes is about 75%, 10% and 15%, respectively.\(^1\) CD4+ (CD3+, CD4+, CD8-) T cells recognize class II MHC (major histocompatibility complex) molecules whereas CD8+ (CD3+, CD4-, CD8+) T cells recognize MHC class I molecules. The CD3 T cell receptor (alpha, beta, gamma, delta) is absent from NK cells. CD56 is the marker which differentiates NK cell from other non-T lymphocytes in humans. There is also a lymphocyte subset called NKT cells. This type expresses both TCR (α and β chains) and NK1.1\(^+\) marker. It is thought to account for 20%-30% of the lymphocyte population in bone marrow and liver and is able to secrete IL-4 as well as INF-γ when activated.

3.1.2 The Role of NK Cells in Innate and Adaptive Immunity

The NK cell is a large granulated lymphocyte customarily defined as ‘a lymphocyte
found in the blood of normal individuals which is capable of lysing tumor cell lines in
the apparent absence of disease, prior sensitization, or deliberate immunization. The
mechanisms by which NK cells function in innate immunity has been well defined. It
has the ability to recognize and induce lysis of target cells, such as infected cells,
tumor cells and allogeneic cells without prior sensitization. In addition, NK cell may
eliminate target cells through antibody-dependent cellular cytotoxicity (ADCC) which
is also involved in adaptive immunity. NK cells are also the source of varieties of
cytokines and chemokines. In addition to its well known role in INF-γ and TNF-α
production in viral infections, it can also secrete IL-5 which may contribute to
eosinophil inflammation.

The proliferation and maturation of NK cell is under the influence of multiple
chemical mediators, including IL-2, IL-15, IL-12 and IL-18. Chemokines have been
proven to play a critical role in NK cell recruitment and activation. These
chemokines include CC chemokines, such as monocyte chemotactic protein-1
(MCP-1), MCP-2, MCP-3, RANTES, macrophage inflammatory protein-1 (MIP-1α),
and MIP-1β; as well as CXC chemokines, such as IL-8 and IP-10. For example, it has
been proven that in invasive Aspergilosis, chemokine-mediated NK cell recruitment
may provide the first line of host defense. When designated CC chemokine ligand-2
(MCP-1/CCL2) neutralizes monocyte chemotactic protein-1, a decreased infiltration
of NK cells is induced, but not in other leukocytes.
There is a complicated interplay between NK cells and professional phagocytes, i.e., neutrophils, macrophages and dendritic cells, either directly or through the role of chemical mediators. Neutrophil derived chemokines have a potential role in NK cell recruitment and activation. NK cells may induce activation of macrophages through the role of INF-γ, whereas IL-12 secreted from macrophages will upregulate NK cell proliferation and maturation. The dendritic cell (DC) is the link between innate and adaptive immunity, acting both as a professional phagocyte and an antigen presenting cell. Through the process of uptake and presentation of an antigen, an immature DC becomes a mature DC, leading to activation of naïve and memory CD4+ and CD8+ T cells. Upon microbial encounter, DC will release IL-2 at an early phase, thus mediating NK cell and B cell activation as well as T cell responses. On the other hand, DC-activated NK cells efficiently kill immature DCs through the NKp30 natural cytotoxicity receptor. In addition, when the NK cell is activated by virus-infected cells with low expression of MHC class I, it will prime the secretion of IL-12 from DC through INF-γ dependent signals. This will result in cytotoxic T lymphocytes (CTL) response. Thus, the innate immune response of NK cell will also lead to an adaptive response.

### 3.1.3 NK Cells in Nasal Polyp and Chronic Sinusitis

Nasal polyp and chronic sinusitis exhibit chronic inflammation. Patients often show recurrent and persistent infection. Although the role of CD4+ and CD8+ T cells has been suggested to contribute to the pathogenesis of nasal polyps and chronic sinusitis,
studies of the role of NK cell and its function in the two diseases are lacking. In normal nasal mucosa, lymphocytes are mainly CD4+ and CD8+ T cells, whereas NK cells were reported to account for less than 2% of the total amount of lymphocytes.\textsuperscript{13} It was reported that in nasal polyps and chronic sinusitis, there was no change in the proportion of NK cells.\textsuperscript{14,15} There are also case reports of patients with dysfunction of NK cells and pansinusitis, or nasal polyps together with recurrent infection.\textsuperscript{16,17} Taken together, although a dysfunction of NK cells may lead to persistent or recurrent infection, there is no study identifying NK cells as an important inflammatory cell in nasal polyps or chronic sinusitis.

3.2 Aim of Study

In chapter 2, we discussed the important role of T cells in the pathogenesis of nasal polyps and chronic sinusitis. An inverse CD4+/CD8+ T cell ratio in nasal polyp or inflamed sinus mucosa compared to controls suggests a T cell disorder. CD8+ T cell may act as a suppressive and a specific cytotoxic T cell against infection. In addition, a previous study reported upregulation of IL-2, which is a growth factor for NK cells in nasal polyp tissue.\textsuperscript{18} The infiltration of the macrophage, an important cell in innate immunity, has been demonstrated in nasal polyps and inflamed sinus mucosa in many studies.\textsuperscript{19-21} These studies as well as our results from the inflammatory cell pattern study (chapter 2) initiated our interest in the role of NK cells in the development of nasal polyps and chronic sinusitis. The aim of our study is to investigate the involvement of NK cells in the chronic inflammation of nasal polyps and chronic
sinusitis; to explore its correlation with other inflammatory cell infiltration, i.e., CD8+ T cells, CD4+ T cells, eosinophils, neutrophils and mast cells; and to explore its correlation with other medical conditions.

3.3 Methodology

3.3.1 Study Patients

Patients with nasal polyps and chronic sinusitis, allergic rhinitis and non-atopic, non-rhinitis controls were randomly selected for this study from the department of Otolaryngology, Head & Neck Surgery in the National University Hospital of Singapore. Working definitions used are shown in chapter 2.3.1. Information of the study groups was summarized in Table 46.

I. Thirteen patients, nine males and four females, aged from 21 to 58 years (mean age 47) with unilateral/bilateral nasal polyps, who were scheduled for functional endoscopic sinus surgery. The diagnosis of nasal polyps was based on medical history and clinical examinations, including nasal endoscopic examination and CT scan.

II. Nine patients, eight males and one female, aged from 20 to 64 years (mean age 38) with unilateral/bilateral chronic sinusitis, who were scheduled for functional endoscopic sinus surgery in our department. The diagnosis of chronic sinusitis was based on medical history and clinical examinations, including nasal endoscopic examination and CT scan.
III. Eleven patients, all males, aged from 13 to 55 years (mean age 28) with allergic rhinitis, who were scheduled for septal surgery in our department. These patients had no history of chronic sinusitis or nasal polyps.

IV. A control group of five non-rhinitis, non-atopic patients, three males and two females, aged from 19 to 68 years (mean age 40), with septal deviation who were scheduled for septal plastic surgery. Patients with nasal polyps, sinusitis, allergic rhinitis and atopy were excluded.

All patients had a trial of intranasal glucocorticosteroids spray but did not show a symptomatic relief of their symptoms. Their medication was discontinued for more than one month prior to the surgery. A signed informed consent was obtained from the study patients before surgery. Approval to conduct this study was granted by the National Medical Research Council of Singapore and the institutional review board of the Medical Faculty of National University of Singapore.

Table 46. Patient groups in the study of natural killer cells.

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Mean age</th>
<th>Number of patients</th>
<th>Male/Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nasal polyps</td>
<td>47</td>
<td>13</td>
<td>9/4</td>
</tr>
<tr>
<td>Chronic sinusitis</td>
<td>38</td>
<td>9</td>
<td>8/1</td>
</tr>
<tr>
<td>Allergic rhinitis</td>
<td>28</td>
<td>11</td>
<td>11/0</td>
</tr>
<tr>
<td>Control patients</td>
<td>40</td>
<td>5</td>
<td>3/2</td>
</tr>
</tbody>
</table>
3.3.2 Method

3.3.2.1 Immunohistochemistry

A nasal polyp tissue/inflamed sinus mucosa biopsy was obtained from all patients with nasal polyps/chronic sinusitis during surgery. One biopsy sample was taken from the middle turbinate of allergic rhinitis and control patients during septal plastic surgery. The specimens were embedded in tissue a freezing medium (Leica Instruments GmbH) in liquid nitrogen immediately after resection. The frozen samples were kept at -80°C for further study. Immunohistochemical staining was applied according to the protocol described in chapter 2.3.3.2. CD56/NCAM-1 Ab-1 (Lab Vision NeoMarker, clone ERIC-1) was used for NK cell staining. Meanwhile, a series of antibodies was used to investigate the involvement of CD4+ and CD8+ T cells, eosinophils, neutrophils sand mast cells. The monoclonal antibodies used for these cells were described in Table 9, chapter2.

To test the specificity of CD56/NCAM-1 Ab-1, immunohistochemical staining of fresh human tonsils by CD56/NCAM-1 Ab-1 together with anti-CD3 (Lab Vision NeoMarker, Rabbit anti-human monoclonal CD3, clone SP7) was applied. The CD56/NCAM-1 Ab-1 was shown to be specific for CD3- NK cell but not for CD3+ NKT cell. Positive cells stained with peroxidase-labeled monoclonal antibody on cell membrane were counted under a light microscope at 400 times magnification. Three areas with high intensity of positive cell distribution were selected in each section. The cell numbers of the three areas were averaged.
3.3.2.2 Allergy Test

Three milliliters of peripheral blood was taken during the surgery. Serum total IgE (tIgE) and specific IgE (sIgE) to a common panel of inhalant allergens, including dust mite (*Dermatophagoides pteronyssinus, Dermatophagoides farinae*), cockroach, common pollen and ragweed mixtures (*Bermuda grass, Ambrosia artemisiifolia, Ambrosia elatior*), common mould and yeast mixtures (*Aspergillus fumigatus, Penicillium notatum, Cladosporium herbarum, Candida albicans, Alternaria tenuis*), and food (egg white, milk, codfish, peanut, soybean) were determined using the ImmunoCAP system. Patients with sIgE \( \geq 0.35 \) IU/ml to at least one of the testing allergens were considered as atopic.

3.3.2.3 Statistics

A standard personal computer with SPSS (Statistical Package for the Social Sciences) 11.5 software (SPSS, Inc., Chicago, Illinois, US) was used for the statistical evaluation of the results. In all the tests, a P value of less than 0.05 was regarded as significant.

I. One-sample t test was used to test the normality of cell counting.

II. Pearson’s correlation was used for the analysis of the correlations between CD56+ NK cells and other inflammatory cells, i.e., CD4+ and CD8+ T cells, eosinophils, neutrophils and mast cells; and of the correlations between NK cells and tIgE or sIgE to common allergens tested. A correlation coefficient above 0 was taken to be a positive correlation; 0-0.3
a weak correlation, 0.3-0.5 a medium correlation, and above 0.5 a strong

correlation.

III. Mann-Whitney test was used to compare the infiltration of NK cells with
the infiltration of other inflammatory cells in the same sample; the NK cell
numbers in patients with and without atopy; and the NK cell numbers in
patients in different study groups, i.e., nasal polyps, chronic sinusitis,
allergic rhinitis patients and controls.

3.4 Results

3.4.1 Allergy test

All of our study patients were Asians. In the nasal polyp group, there were seven
Chinese, two Malays, three Indians and one Philippino. In the chronic sinusitis group,
there were one Indian and eight Chinese. In the allergic rhinitis group, there were
seven Chinese, three Indians and one Malay. In the control group, there were three
Chinese, one Malay and one Indian.

All the patients in the nasal polyp, chronic sinusitis and allergic rhinitis groups made
serum available for allergy test. In the control group, serum was only made available
by three patients. The percentage of patients with high levels of total serum IgE (tIgE
≥100 IU/ml) and atopy (diagnosis criteria: at least has one serum specific IgE ≥0.35
IU/ml to the common allergens tested) is shown in Table 47.
Table 47. Percentage of a high level of tIgE (tIgE ≥ 100 IU/ml) and atopy of nasal polyp patients (n=13), chronic sinusitis patients (n=9), allergic rhinitis patients (n=11) and controls (n=3).

<table>
<thead>
<tr>
<th>Group</th>
<th>Total IgE (≥100 IU/ml)</th>
<th>Atopy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nasal polyp</td>
<td>5 (38.5%)</td>
<td>5 (38.5%)</td>
</tr>
<tr>
<td>Chronic sinusitis</td>
<td>5 (55.6%)</td>
<td>4 (44.4%)</td>
</tr>
<tr>
<td>Allergic rhinitis</td>
<td>8 (72.7%)</td>
<td>11 (100%)</td>
</tr>
<tr>
<td>Controls</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

3.4.2 Specificity Control

Figure 30. Immunohistochemistry staining of a human tonsil with anti-CD56 and anti-CD3 antibodies (light microscope 100 times magnification). A. Staining with anti-CD56. B. Staining with anti-CD3.
Figure 30 shows the immunohistochemistry staining of CD56/NCAM-1 Ab-1 in a human tonsil. By comparing this with anti-CD3 staining, it was confirmed that CD56/NCAM-1 Ab-1 used in our study was CD3 negative. The cell type we studied was NK cell (CD56+CD3-) but not NKT cell which is CD3+.

3.4.3 Correlation of NK Cell with tIgE and sIgE

Pearson’s correlation analysis showed that there was no significant correlation between NK cell numbers in the nasal polyp tissue/inflamed sinus mucosa and tIgE or sIgE to the common allergens tested.

3.4.4 NK Cell and Other Inflammatory Cells in the Same Sample

3.4.4.1 Mean and 95% Confidence Interval

Table 48. Median and 95% confidence interval (mean±SD) of the cell number of NK cells, CD4+ and CD8+ T cells, eosinophils, neutrophils and mast cells in nasal polyp tissue (n=13), inflamed sinus mucosa (n=9), middle turbinate from allergic rhinitis patients (n=11) and controls (n=5).

<table>
<thead>
<tr>
<th></th>
<th>NK</th>
<th>CD4+ T cell</th>
<th>CD8+ T cell</th>
<th>Eosinophil</th>
<th>Neutrophil</th>
<th>Mast cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyp tissue</td>
<td>12</td>
<td>32</td>
<td>46</td>
<td>25</td>
<td>13</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>(15.2±14.7)</td>
<td>(33.4±23.1)</td>
<td>(48.9±26.7)</td>
<td>(21.9±15.8)</td>
<td>(18.2±15.6)</td>
<td>(8.2±6.7)</td>
</tr>
<tr>
<td>Inflamed sinus</td>
<td>2</td>
<td>16</td>
<td>15</td>
<td>7</td>
<td>28</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>(5.8±8.6)</td>
<td>(17.9±14.0)</td>
<td>(31±30.6)</td>
<td>(11±9.2)</td>
<td>(35.9±41.3)</td>
<td>(11.2±10.5)</td>
</tr>
<tr>
<td>MT (AR)¹</td>
<td>3</td>
<td>18</td>
<td>34</td>
<td>2</td>
<td>18</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>(7.1±13.1)</td>
<td>(30.6±32.7)</td>
<td>(48.6±59.7)</td>
<td>(10.3±17.6)</td>
<td>(22.6±23.5)</td>
<td>(8.3±4.8)</td>
</tr>
<tr>
<td>MT (CON)²</td>
<td>3</td>
<td>18</td>
<td>21</td>
<td>3</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>(2.2±1.1)</td>
<td>(25.6±27.6)</td>
<td>(27±22.6)</td>
<td>(10±15.3)</td>
<td>(18.4±21.5)</td>
<td>(9.4±5.9)</td>
</tr>
</tbody>
</table>

MT (AR)¹, middle turbinate from allergic rhinitis. MT (CON)², middle turbinate from controls.
Table 48 shows the median and 95% confidence interval of the number of NK cells, CD4+ and CD8+ T cells, eosinophils, neutrophils and mast cells in nasal polyp tissue, inflamed sinus mucosa and middle turbinate mucosa from allergic rhinitis and controls. Nasal polyp tissues had the highest median and mean number of NK cells in all the study groups. There were similar levels of the median and mean of NK cell numbers in chronic sinusitis patients, allergic rhinitis patients and controls. Since the role of CD4+ and CD8+ T cells, eosinophils, neutrophils and mast cells was discussed previously in chapter 2, we will not analyze their infiltration in this section any further.

3.4.4.2. NK Cell Infiltration Compared to Other Inflammatory Cells in the Same Sample

Table 49. P values of Mann-Whitney test for the cell number of NK cells and other inflammatory cells (CD4+ and CD8+ T cells, eosinophils, neutrophils and mast cells) in nasal polyp tissue (n=13), inflamed sinus mucosa (n=9), middle turbinate from allergic rhinitis patients (n=11) and controls (n=5).

<table>
<thead>
<tr>
<th></th>
<th>Polyp tissue</th>
<th>Inflamed sinus mucosa</th>
<th>MT (AR) ¹</th>
<th>MT (CON) ²</th>
</tr>
</thead>
<tbody>
<tr>
<td>NK-CD4+ T cell</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>NK-CD8+ T cell</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>NS</td>
</tr>
<tr>
<td>NK-Eosinophil</td>
<td>NS ³</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>NK-Neutrophil</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>NK-Mast cell</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.05</td>
<td>NS</td>
</tr>
</tbody>
</table>

MT (AR) ¹, middle turbinate from allergic rhinitis. MT (CON) ², middle turbinate from controls. NS ³, no significant difference.
Table 49 shows the P value of Mann-Whitney test of cell number between NK cells and other inflammatory cells, i.e., CD4+ and CD8+ T cells, eosinophils, neutrophils and mast cells, in all the study groups. In nasal polyp tissue and inflamed sinus mucosa, the NK cell number was significantly lower than the CD4+ and CD8+ T cell numbers. However, there was no significant difference between the cell counts of NK cells and the cell counts of eosinophils, neutrophils or mast cells. In the middle turbinate mucosa from allergic rhinitis patients, there were significantly higher levels of CD4+ and CD8+ T cells and mast cells than of NK cells. There was no significant difference between the NK cell level and the eosinophil or neutrophil levels in this group. In controls, no significant difference was identified between NK cell level and other inflammatory cell levels.

3.4.4.3 Correlation of NK cells and Other Inflammatory Cells

Table 50. P value and correlation coefficient of significant Pearson’s correlations between NK cell level and other inflammatory cell levels (CD4+ and CD8+ T cells, eosinophils) in middle turbinate mucosa of allergic rhinitis patients.

<table>
<thead>
<tr>
<th></th>
<th>P value</th>
<th>Correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>NK-CD4+ T cell</td>
<td>&lt;0.05</td>
<td>0.666</td>
</tr>
<tr>
<td>NK-CD8+ T cell</td>
<td>&lt;0.0001</td>
<td>0.955</td>
</tr>
<tr>
<td>NK-Eosinophil</td>
<td>&lt;0.0001</td>
<td>0.925</td>
</tr>
</tbody>
</table>

No significant correlation between the NK cell level and other inflammatory cell levels was identified in nasal polyp tissue, inflamed sinus mucosa and middle turbinate mucosa from controls. However, in the middle turbinate mucosa of allergic rhinitis patients, there was a significant, positive and strong correlation between the
NK cell level and the CD4+ T cell, CD8+ T cell and eosinophil levels. The P values and correlation coefficients are shown in Table 50.

3.4.5 NK Cells in Patients with and without Atopy

No significant difference was identified between NK cell levels in nasal polyp/inflamed sinus mucosa in patients with and without atopy.

3.4.6 NK Cells in Different Study Groups

Figure 31. NK (CD56+CD3-) cell immunohistochemical staining in nasal polyp tissues (A), inflamed sinus mucosa (B), middle turbinate from allergic rhinitis patients (C) and controls (D). (Light microscope 100 times magnification).
**Figure 31**, Continued.

**Figure 31** shows CD56 monoclonal antibody staining in nasal polyp tissue, inflamed sinus mucosa, and middle turbinate mucosa from allergic rhinitis and controls. NK cells could be found in the epithelium, subepithelium and deep lamina propria. NK cells were mainly distributed beneath the epithelium with clusters in nasal polyp tissue and inflamed sinus mucosa. Infiltration into the epithelium was also commonly found. In the middle turbinate mucosa from allergic rhinitis and controls, NK cells were more prone to distribute themselves in deep lamina propria as single cells. The character of this distribution was different from that of the CD4+ and CD8+ T cells,
which were often distributed diffusely in the nasal polyp tissue or inflamed sinus mucosa. Sometimes, but not always, NK cells clustered in these same areas with high neutrophil infiltration.

Statistical analysis showed that the nasal polyp tissue had significantly higher NK cell numbers than the inflamed sinus mucosa, and the middle turbinate mucosa from allergic rhinitis and controls. There was no significant difference in the NK cell numbers in the inflamed sinus mucosa, and in the middle turbinate of allergic rhinitis and controls. Z values and the P values of Mann-Whitney test are shown in Table 51.

**Table 51.** Z value and P value (2-tailed) of Mann-Whitney test of NK cells in nasal polyp tissue (n=13), inflamed sinus mucosa (n=9), middle turbinate from allergic rhinitis patients (n=11) and controls (n=5).

<table>
<thead>
<tr>
<th></th>
<th>Z value</th>
<th>P value (2-tailed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nasal polyp tissue</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NK (NP)(^1)-NK(SI)(^2)</td>
<td>2.218</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>NK(NP)-NK(AR)(^3)</td>
<td>2.644</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>NK(NP)-NK(CON)(^4)</td>
<td>2.822</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Inflamed sinus mucosa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NK(SI)-NK(AR)</td>
<td>0.776</td>
<td>NS(^5)</td>
</tr>
<tr>
<td>NK(SI)-NK(CON)</td>
<td>0.071</td>
<td>NS</td>
</tr>
<tr>
<td>MT(AR)(^5) and MT (CON)(^6)</td>
<td>0.872</td>
<td>NS</td>
</tr>
</tbody>
</table>

NK (NP)\(^1\), NK cell in nasal polyp tissue. NK(SI)\(^2\), NK cell in inflamed sinus mucosa. NK(AR)\(^3\), NK cell in middle turbinate from allergic rhinitis. NK(CON)\(^4\), NK cell in middle turbinate from controls. MT(AR)\(^5\), middle turbinate from allergic rhinitis. MT (CON)\(^6\), middle turbinate from controls. NS\(^5\), no significant difference.

**Figure 32** is a scatter figure of NK cells in nasal polyp tissue, inflamed sinus mucosa and middle turbinate from allergic rhinitis and controls. P value of Mann-Whitney test with significance is indicated. The NK cell level in nasal polyp tissue was
significantly higher than that in inflamed sinus mucosa, and middle turbinate from allergic rhinitis and controls.

**Figure 32.** Scatter figure of NK cells (CD56+) in nasal polyp tissue (n=13), inflamed sinus mucosa (n=9), middle turbinate from allergic rhinitis patients (n=11) and controls (n=5). P, P value of Mann-Whitney test.

### 3.4.7 Percentage of NK Cells in Total Lymphocytes in Different Study Groups

In addition to the analysis regarding absolute numbers of cells, we further analyzed relative numbers of cells, i.e., the lymphocyte subsets in the study groups, including CD4+ T cells, CD8+ T cells and NK cells. Because B cells were rarely seen in all of the groups, their involvement ignored. CD8+ T cells were prominent over CD4+ T cell and NK cell numbers in nasal polyps, inflamed sinus mucosa and middle turbinate mucosa of allergic rhinitis. Whereas in the middle turbinate mucosa from
controls, the average level of CD4+ T cells was slightly higher than CD8+ T cells. These findings were in agreement with the results in chapter 2. The mean percentages of NK cells in total lymphocytes in nasal polyp tissue, inflamed sinus mucosa, middle turbinate mucosa from allergic rhinitis and controls were 16%, 15%, 9% and 10%, respectively. **Figure 33** is the stacked bar chart of the mean percentages of the lymphocyte subsets, i.e., CD4+ and CD8+ T cells and NK cells in nasal polyp tissue, inflamed sinus mucosa, and middle turbinate mucosa from allergic rhinitis patients and controls.

![Stacked bar chart of the mean percentages of the lymphocyte subsets (CD4+ and CD8+ T cells and NK cell) in nasal polyp tissue (n=13), inflamed sinus mucosa (n=9), and middle turbinate mucosa from allergic rhinitis patients (n=11) and controls (n=5).](image)

**Figure 33.** Stacked bar chart of the mean percentages of the lymphocyte subsets (CD4+ and CD8+ T cells and NK cell) in nasal polyp tissue (n=13), inflamed sinus mucosa (n=9), and middle turbinate mucosa from allergic rhinitis patients (n=11) and controls (n=5).

### 3.5 Discussion

In our study, we evidenced that lymphocytes, especially CD8+ T cells, may play a central role in the pathogenesis of nasal polyps and chronic sinusitis, as discussed in
**Chapter 2.** Although NK cell infiltration was not comparable to CD8+ T cell infiltration, the similarity in the levels of NK cells, with eosinophils and neutrophils in nasal polyps and inflamed sinus mucosa gives an indication of the contribution of NK cell to the pathogenesis. In addition, the NK cell level in the nasal polyp tissue was significantly higher than that in the inflamed sinus mucosa, and middle turbinate of allergic rhinitis and controls. This suggests that different mechanism may be involved. When compared with the percentage of NK cell in the total lymphocytes (CD4+ and CD8+ T cells and NK cell) in different subjects, we identified increased percentages in the nasal polyp tissue and inflamed sinus mucosa (16% and 15% respectively) to that in the middle turbinate mucosa from allergic rhinitis and controls (9% and 10% respectively).

NK cells are commonly regarded as the first line against infection of bacteria, virus and fungus. Patients with NK deficiency may suffer more frequently from infectious diseases.\textsuperscript{24} Studies in recent years suggest that the mechanism of NK cell activation as well as the consequent immune response is far from known. In addition to its role in innate immunity, the NK cell may affect adaptive immunity as well. One way is to regulate T cell function through release of chemical mediators. Another way is to eliminate target cells with altered MHC class II antigens. Also, NK cells activated by virus-infected cells with MHC class I low expression will induce CD8+ T cell recruitment and activation.\textsuperscript{12}
Besides the role of NK cell in infection, it contributes to combat allergy in the airway. A study by Walker et al.\textsuperscript{25} indicated that depletion of NK1.1+ cell (NK cell and NKT cell) will cause a significant inhibition of eosinophil infiltration (>50%) together with a reduction of IL-5 in a murine model challenged with ragweed. Although T cells also play an important role in allergic inflammation as well as in IL-5 production, it was suggested that the NK cell may exert its role separately. In another study of allergic asthma by Korsgren et al.,\textsuperscript{26} the murine model with depletion of NK1.1+ cells showed inhibition of the infiltration of eosinophils and CD3+ T cells. The secretion of a number of cytokines, including IL-4, IL-12 and IL-5 was also inhibited. Further results showed that it was NK cell, but not NKT cell, that played the central role. It was also suggested that increased NK activity may predispose to a higher risk of developing allergic inflammation.

Although the biology of the NK cell has been widely studied in recent years, there are very few reports about its role in the pathogenesis of nasal polyps and chronic sinusitis. Actually, in diseases correlated with the presence of NK cell in the upper airway, the most commonly reported ones are NK/T-cell lymphoma, related with chronic Epstein-Barr virus infection,\textsuperscript{27} and chronic infectious rhinitis.\textsuperscript{28} This is in agreement with our finding that there was no difference in NK cell number and NK cell percentage in the middle turbinate mucosa from persistent allergic rhinitis patients and controls. In a study Sanchez-Segura et al.\textsuperscript{14} it was reported that cellular infiltration in nasal polyp tissue mainly consisted of T cells (over 80%), especially
CD8+ T cells. B cells and NK cells accounted for about 5% each in the total lymphocyte count. Compared with the peripheral blood, the pan T cell level in nasal polyps was significantly higher, while the NK cell level was significantly lower. In the study of chronic sinusitis, a similar finding was reported.\textsuperscript{15} It is interesting that our study patients showed an increased percentage of NK cells in nasal polyp tissue and inflamed sinus mucosa, as compared to the middle turbinate from allergic rhinitis patients and controls, which has thus far not been reported in the literature.

The NK cell distribution in our study groups often correlated well with the distribution of neutrophils. In nasal polyp tissue and inflamed sinus mucosa, neutrophils and NK cells were mainly distributed in the subepithelium area and formed small clusters. NK cell infiltration of the epithelium was commonly identified as well. In the middle turbinate mucosa from allergic rhinitis patients and controls, both neutrophils and NK cells were prone to distribute themselves in the deep lamina propria rather than in the subepithelium. The neutrophil is a professional phagocyte, which plays an important role in innate immunity. Varieties of chemokines produced by neutrophils,\textsuperscript{29} including macrophage inflammatory protein 1α (MIP-1α), MIP-1β, and INF-γ inducible protein 10 (IP-10) have been proved to be chemotactic and activators of NK cells.\textsuperscript{4,5} It has been suggested that both neutrophils and NK cells play important roles in early microorganism infection. The infection will recruit NK cells to the inflammatory foci. Depletion of NK cells in animal models may cause continuous recruitment of neutrophils but non-effective clearance of pathogens,
leading to pathological changes and increased mortality. These findings indicate the important role of NK cells against infection.

Although NK cells and neutrophils were often distributed in similar areas in our patients, in some cases, they showed disparate distributions. In addition, there was no significant correlation identified between cell numbers of neutrophils and NK cells. Therefore, although an infection may induce NK cell recruitment, it seems to play a partial role only. Varieties of chemokines have been suggested to play important roles in NK cell recruitment and activation, in which RANTES and IL-8 upregulation in nasal polyps and chronic sinusitis has been commonly identified. IL-8 is synthesized by macrophages, lymphocytes, neutrophils and structural cells and is closely correlated with nasal neutrophilia. RANTES has been shown to be released from eosinophils and epithelial cells in nasal polyps, playing a role as a potent chemoattractant of eosinophils and exerting chemotactic activity on them. Thus, the recruitment of NK cells caused by chemokines is not only related with neutrophils but also with eosinophils, epithelial cells and other inflammatory or structural cells in nasal polyps and chronic sinusitis. The expression of IL-8 and RANTES by epithelial cells of nasal polyp tissue may explain the infiltration of NK cells in the epithelium in our study patients.

In addition to the activation by infected cells, NK cell differentiation and proliferation is under the control of cytokines, i.e., IL-2, IL-15, IL-12 and IL-18. In nasal polyps
and chronic sinusitis, an increased level of IL-12 and IL-2 has been identified. Whether they play important roles in NK cell proliferation in nasal polyps and chronic sinusitis is not known. The upregulation of NK cells in nasal polyps and chronic sinusitis may have effects not only on innate immunity but also on adaptive immunity through direct interactions between cells or indirect interactions regulated by chemical mediators. By lysis of dendritic cells and macrophages, NK cells may affect antigen presentation. Recently, it was reported that NK cells can enhance proliferation and activation of CD4+ and CD8+ T cells in response to specific Ag and CD3 cross-linking through the interaction of 2B4 (CD244) on NK cells and CD48 on T cells. The 2B4/CD48 interaction between NK cells will enhance their cytotoxicity and INF-γ production induced by IL-2. The cytokines released by NK cells include INF-γ, TNF-α, GM-CSF, and IL-5. Through the roles of these cytokines, NK cells will regulate not only antimicrobial infections but also allergic inflammation. These effects may lead to a response by cytotoxic T lymphocytes (CTL) or a response by eosinophils leading to allergic inflammation.

Taken together, our study identified an increased percentage of NK cells in nasal polyps and inflamed mucosa, as compared with the middle turbinate from allergic rhinitis patients and controls. In addition, the nasal polyp tissue had a significantly higher number of NK cells than the inflamed sinus mucosa, and the middle turbinate from allergic rhinitis and controls. To the best of our knowledge, this is the first report of the importance of NK cells as important inflammatory cells in the pathogenesis of
nasal polyp and chronic sinusitis. In addition to the correlation with neutrophils in some cases, NK cells also showed a different distribution in other cases, suggesting infection may only play a partial role in NK cell response. The role of chemokines, such as RANTES and IL-8, in NK cell recruitment and activation in nasal polyps and chronic sinusitis needs to be further clarified. The complicated roles played by NK cells in innate and adaptive immunity has attracted attention in recent years. Besides its role in innate immunity, NK cell may regulate adaptive immunity through interactions with other cells or effects mediated by cytokines, affecting both the T cell and eosinophil response. Whether the increased percentage of NK cells in nasal polyps and chronic sinusitis is correlated with eosinophilia and CD8+ T cell infiltration remains to be clarified further. It may provide important information for understanding the underlying mechanisms.

Reference List

5. Maghazachi AA, al Aoukay A, Schall TJ. C-C chemokines induce the


Chapter 4  
Evaluating the Association of IgE-Mediated Allergy with the 
Pathogenesis of Nasal Polyps and Chronic Sinusitis by an Immunodot Blot Array 

System

4.1 Testing for IgE-Mediated Allergy, a Review

4.1.1 Allergy and IgE

It is not until last century that the word ‘allergy’ we commonly use now appeared. In
1906, Clemens von Pirquet proposed the term ‘allergy’ to denote changed activity. It is
derived from the Greek in which “allos” means different or changed and ‘ergos’ means
work or action. In 1923, Robert A. Cooke and Arthur Fernandez Coca proposed the term
‘atopy’ to denote clinic allergy. The proposed definition read ‘the individuals as a group
have a peculiar capacity to become sensitive to certain proteins to which their
environment and habits of life frequently expose them’. Later, other factors, such as a
positive skin test were also applied.

In immunology, there are four types of allergies or hypersensitivities. Type I is IgE-
mediated allergy characterized by mast cell activation caused by IgE cross-linking. It is
also called “immediate reaction” because of the very short duration of the reaction,
lasting only minutes. Type II is a cytotoxic reaction in which cell-bound antigens interact
with the circulating IgG or IgM. They activate the complement cascade which results in
lysis of the cell. Type III is an immune complex reaction. Circulating antigens and
antibodies form complexes which activate the complement cascade. Then inflammatory
cells such as neutrophils will infiltrate. Type IV is a cellular immune reaction which is
also called delayed type hypersensitivity for its onset of symptoms after 24-48 hours of
allergen exposure. In this reaction, the antigen becomes a part of the target cell. When it
is recognized by the cytotoxic T cell, which has specific receptors for the antigen, it will cause lysis of the target cell. In the meantime, the sensitized T cells can release a pattern of cytokines which cause inflammation and tissue damage.

In the last two decades, a tendency to use the word “allergy” or “hypersensitivity” to describe all kinds of unexpected reactions in the skin and mucosal surfaces has been developed. The term hypersensitivity has been redefined as follows in EAACI (European Academy of Allergology and Clinical Immunology) in 2001 as follows: “Hypersensitivity causes objectively reproducible symptoms or signs, initiated by exposure to a defined stimulus at a dose tolerated by normal subjects.”

The term hypersensitivity was proposed to be classified into: allergic hypersensitivity when immunologic mechanism is defined or strongly suspected; and nonallergic hypersensitivity when immunologic mechanism is excluded. Allergic hypersensitivity can be IgE-mediated and not-IgE mediated. Diseases related with IgE-mediated diseases include seasonal allergic rhinitis or asthma, while contact dermatitis is non-IgE mediated allergic disease.

In clinical research, the term ‘allergy’ we use mostly refers to the type I IgE-mediated hypersensitivity. Pepys first termed IgE-mediated allergic reactions to inhalant allergens as “atopic allergy” in the 1970s. The term “atopic” was then used synonymously with “IgE-mediated”. EAACI has proposed the definition of atopy as follows: “Atopy is a personal or familial tendency to produce IgE antibodies in response to low doses of allergens, usually proteins, and to develop typical symptoms such as asthma, rhinoconjunctivitis, or eczema/dermatitis.”
IgE has been known to play an important role in allergy, however, whether we can use IgE as measurement in the diagnosis of allergy remains uncertain. It has been suggested that the serum total IgE is correlated to the following factors.²

1. The state of atopy.
2. The number and degree of specific allergens.
3. The degree and duration of allergen exposure.
4. The organs involved. It is proposed that total IgE level is well correlated with the severity of dermatitis. However, in patients who have airway symptoms, the correlation is very poor.

The normal IgE level for adults is defined as <100 IU/ml, however, it is suggested that different populations under study may have widely varying levels. Especially in populations with a genetic predisposition, the total IgE levels are prone to be higher than for others, even when symptoms are absent.

In the study to address the relationship between IgE and allergy, we have to look into the molecular processes underlying IgE synthesis. During an immune response, there are two types of signals which will switch B cells to synthesize IgE. The first depends on the cytokine and the second results from DNA recombination. It is well known that IL-4 is the crucial cytokine for IgE production.³ Later, IL-13 was demonstrated to direct naive human B cells to switch to IgG4 and IgE production.⁴

The interaction between CD40L expressed on T cells and CD40 on B cells provides the second signal for IgE synthesis. Allergen specific T cells are required for the initiation of
IgE synthesis. Cells which have the function of IL-4 and IL-13 secretion, such as basophils and mast cells, will contribute to non-T cell dependent IgE amplification which is nonspecific polyclonal IgE. Genetic predisposition and environmental factors are of great importance to determine Th2-dependent IgE synthesis. The nature of an allergen together with the patient’s genetic background will drive the immune response towards a Th1 or Th2 response. Environmental factors, such as bacterial secreted endotoxin and lipopolysaccharide (LPS), are also important in host response. Early exposure to LPS before allergen challenge has been proved to inhibit sensitization, while on the other hand, exposure after allergen challenge will exacerbate the sensitization. 

4.1.2 Diagnosis of Allergy

4.1.2.1 In vivo tests

The skin prick test is the main in vivo test for IgE-mediated allergy. IgE-mediated allergic reaction in the test is a wheal-and-flare reaction. It is an irregular, blanched, elevated wheal appears, surrounded by an area of erythema (flare). This reaction shows after 5 minutes and peaks at 30 minutes of allergen injection and is defined as an immediate reaction. Inconstantly, immediate reaction is followed by a late-phase reaction (LPR) which shows after 1 to 2 hours and peaks at 6 to 12 hours after injection. Immediate reaction is mediated by IgE triggered histamine release. However, the size of wheal-and-flare reaction usually does not correlate with concentration of histamine released. Other neurogenic mediators, such as substance P, neurokinin A and calcitonin gene-related peptide are suggested to interact with cellular inflammatory components in the generation of immediate reaction. The mechanism underlying LPR is not well understood. Mast cell derived chemical mediators may regulate the infiltration of leukocytes, for example,
CD4+ and CD8+ T cells and eosinopils in LPR.\textsuperscript{6} The skin prick test will provide a fast and simple diagnosis at low cost. However, there is no close correlation between symptoms and IgE-mediated allergy. In recent years, intradermal dilutional testing (IDT) has been applied in the diagnosis of allergy, especially allergies related with inhalant allergens. The IDT was reported to be more sensitive than the prick test.\textsuperscript{7} There are multiple factors affecting the result of a skin prick test, including standardization of allergens, applied area, age, gender, race, season, drug usage etc.\textsuperscript{8}

4.1.2.2 \textbf{In vitro tests}

The radioallergosorbent test (RAST) has been as widely used as the in vitro test for IgE-mediated allergy. Other tests, such as leucocyte stimulation index (SI), IL-4 production, IgE RAST, histamine release test (HRT), leukotriene release test (LRT) and basophil activation test (BAT), test of hymenoptera venom-specific IgG antibody, IgG precipitins for organic dusts, mast cell tryptases, and the venom RAST inhibition test may also be applied to obtain helpful information. A recently developed multiallergen IgE screening assay has provided a simpler way for more extensive tests of indoor and outdoor allergens.\textsuperscript{9} Compared to in vitro testing, the skin test is more sensitive but less specific.\textsuperscript{8} The correlation between in vivo and in vitro tests ranges from 85\% to 95\% which may depend on the nature of allergens.\textsuperscript{8} Clinical history has to be considered in the diagnosis of allergy.

4.2 \textbf{Aim of Study}

In \textit{chapter 1}, we briefly reviewed the association of allergy in the pathogenesis of nasal
polyps and chronic sinusitis. Whether allergy plays a cause-and-effect role in the pathogenesis of nasal polyps and chronic sinusitis remains controversial. In clinic, the skin prick test and the RAST are most commonly used to diagnose atopy. There are controversial reports on whether the incidence of a positive test is higher in nasal polyp or chronic sinusitis patients than that in controls.\textsuperscript{10-12}

We have identified a remarkable eosinophil infiltration in both nasal polyp tissue and inflamed sinus mucosa, which was discussed in \textit{chapter 2}. However, we were not able to identify a higher incidence of positive ImmunoCAP test in the patients, as compared to that in the general population. So far, no more than 10 allergen extracts have been accepted by WHO (World Health Organization) as international standards. This may limit the diagnosis greatly because there may be hundreds of allergens having clinical importance. In this section, we will introduce a preliminary study of a multiple allergen immunodot blot test. The aim of our study is to develop a simple test for the detection of specific IgE to multiple allergens, especially the non-commonly tested allergens. This may provide important information in the diagnosis of allergy and understanding its role in the pathogenesis of nasal polyps and chronic sinusitis.

4.3 Methodology

4.3.1 Study Patients

Four groups of patients are included in this study. Their information is summarized in Table 52.
I. Ten nasal polyp patients, seven males and three females, aged from 12 to 52 years (mean age 43).

II. Ten chronic sinusitis patients, six males and four females, aged from 31 to 72 years (mean age 46).

III. Forty-seven patients with allergic rhinitis, 34 males and 13 females, aged from 18 to 57 years (mean age 26).

IV. A control group of thirteen patients with nonallergic rhinitis, five males and eight females, aged from 21 to 49 years (mean age 36).

All the patients were randomly selected from the department of Otolaryngology, National University Hospital of Singapore. The criteria of diagnosis were the same as those mentioned in chapter 2.

Table 52. Patient groups in the study of immunodot blot array system.

<table>
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<th>Patient group</th>
<th>Mean age</th>
<th>Number of patients</th>
<th>Male/Female</th>
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<td>10</td>
<td>7/3</td>
</tr>
<tr>
<td>Chronic sinusitis</td>
<td>46</td>
<td>10</td>
<td>6/4</td>
</tr>
<tr>
<td>Allergic rhinitis</td>
<td>26</td>
<td>47</td>
<td>34/13</td>
</tr>
<tr>
<td>Control patients</td>
<td>36</td>
<td>13</td>
<td>5/8</td>
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</tbody>
</table>

4.3.2 Immunoarray System

4.3.2.1 Preparation of Allergens

185 allergens were used in the immunoarray study, including common allergens such as dust mite, mold, pollen, food etc. Skin prick extracts from ALK-Abelló S.A. (Spain) were purchased. Concentration characterization showed that many of these extracts did not reach the minimum concentration of 0.2 mg/ml required in our study. To solve this
problem, raw materials were collected locally or purchased from Greer Laboratories Inc. (Greer Labs Inc, Lenoir, NC, USA) for protein extraction. In summary, there were 120 skin prick extract solutions available from ALK-Abelló S.A. The other 65 allergens were in the condition of raw materials ready for protein extraction. Allergens used are listed in Table 53. They were homogenized in liquid nitrogen in a mortar until a fine extraction solution with Phosphate-buffered saline and 20% glycerol (v/v) was formed. The solutions were kept at 4°C overnight. The next morning, the solutions were centrifuged for 5 minutes at a speed of 14,000g in 4°C until a clear supernatant came out. The supernatants were kept at 4°C for further use. A Microassay (BioRad) system was used to characterize the concentrations of the protein extracts to ensure a concentration of 0.2 µg/µl.

**Table 53.** Allergens used in immunoarray dot blot system. Allergen extract solutions for skin prick test were all from ALK-Abelló S.A. (Spain), except for: 1, samples locally collected (local mites were cultured under natural condition and harvested by modified Tullgren funnel); 2, samples purchased from Greer Laboratory Inc. and extracted.

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<tr>
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<td><strong>Agropyron repens</strong> (Quack grass)</td>
</tr>
<tr>
<td></td>
<td><strong>Alopecurus pratensis</strong> (Foxtail, meadow)</td>
</tr>
<tr>
<td></td>
<td><strong>Alnus glutinosa</strong> (Alder, black)</td>
</tr>
<tr>
<td></td>
<td><strong>Amaranthus hybridus</strong> (Careless, weed)</td>
</tr>
<tr>
<td></td>
<td><strong>Ambrosia artemisiifolia</strong>&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td><strong>Ambrosia trifida</strong> (Ragweed, tall)</td>
</tr>
<tr>
<td></td>
<td><strong>Anthoxanthum odoratum</strong> (Vernal grass, sweet)</td>
</tr>
<tr>
<td></td>
<td><strong>Artemisia vulgaris</strong> (Mugwort, common)</td>
</tr>
<tr>
<td>Category</td>
<td>Name</td>
</tr>
<tr>
<td>----------</td>
<td>------</td>
</tr>
<tr>
<td>Pollen</td>
<td><em>Arecastrum romanzoffianum</em> (Palm, Queen)</td>
</tr>
<tr>
<td></td>
<td><em>Atriplex polycarpa</em> (Allscale)</td>
</tr>
<tr>
<td></td>
<td><em>Avena sativa</em> (Oats, cultivated)</td>
</tr>
<tr>
<td></td>
<td><em>Baccharis halimifolia</em> (Baccharis, eastern)</td>
</tr>
<tr>
<td></td>
<td><em>Baccharis sarothroides</em> (Baccharis, western)</td>
</tr>
<tr>
<td></td>
<td><em>Betula pendula</em> (^2)</td>
</tr>
<tr>
<td></td>
<td><em>Betula verrucosa</em> (Birch, white)</td>
</tr>
<tr>
<td></td>
<td><em>Brassica Spp.</em> (^2)</td>
</tr>
<tr>
<td></td>
<td><em>Bromus mollis</em> (Spear grass)</td>
</tr>
<tr>
<td></td>
<td><em>Carpinus betulus</em> (Hornbeam)</td>
</tr>
<tr>
<td></td>
<td><em>Casuarina equisetifolia</em> (^2)</td>
</tr>
<tr>
<td></td>
<td><em>Calluna vulgaris</em> (Heather)</td>
</tr>
<tr>
<td></td>
<td><em>Chenopodium album</em> (Lamb's quarter)</td>
</tr>
<tr>
<td></td>
<td><em>Chrysanthemum leucanthemum</em> (Daisy, Ox eye)</td>
</tr>
<tr>
<td></td>
<td><em>Corn flour</em></td>
</tr>
<tr>
<td></td>
<td><em>Corylus avellana</em> (Hazel)</td>
</tr>
<tr>
<td></td>
<td><em>Cryptomeria japonica</em> (Cedar, Japan)</td>
</tr>
<tr>
<td></td>
<td><em>Cynodon dactylon</em> (Bermuda grass)</td>
</tr>
<tr>
<td></td>
<td><em>Cynosurus cristatus</em> (Dog's tail grass)</td>
</tr>
<tr>
<td></td>
<td><em>Cupressus arizonica</em> (Cypress, Arizona)</td>
</tr>
<tr>
<td></td>
<td><em>Cupressus sempervirens</em> (Cypress)</td>
</tr>
<tr>
<td></td>
<td><em>Cynodon dactylon</em> (Bermuda grass) (^2)</td>
</tr>
<tr>
<td></td>
<td><em>Dactylis glomerata</em> (Orchard grass)</td>
</tr>
<tr>
<td></td>
<td><em>Dahlia cultorum</em> (Dahlia)</td>
</tr>
<tr>
<td></td>
<td><em>Eucalyptus globules</em> (^2)</td>
</tr>
<tr>
<td></td>
<td><em>Fagus sylvatica</em> (Beech, European)</td>
</tr>
<tr>
<td></td>
<td><em>Festuca pratensis</em> (Fescue, meadow)</td>
</tr>
<tr>
<td></td>
<td><em>Fraxinus excelsior</em> (Ash)</td>
</tr>
<tr>
<td></td>
<td><em>Holcus lanatus</em> (Velvet grass)</td>
</tr>
<tr>
<td></td>
<td><em>Hordeum vulgare</em> (Barley, activated)</td>
</tr>
<tr>
<td></td>
<td><em>Humulus lupulus</em> (Hops)</td>
</tr>
<tr>
<td></td>
<td><em>Juniperus ashei</em> (Cedar, Moutain)</td>
</tr>
<tr>
<td></td>
<td><em>Ligustrum vulgare</em> (Privet, common)</td>
</tr>
<tr>
<td></td>
<td><em>Lolium perenne</em> (Rye grass, perennial)</td>
</tr>
<tr>
<td></td>
<td><em>Medicago sativa</em> (Alfalfa)</td>
</tr>
<tr>
<td></td>
<td><em>Olea europaea</em> (Olive)</td>
</tr>
<tr>
<td></td>
<td><em>Oil Palm Pollen</em> (^1)</td>
</tr>
<tr>
<td>Category</td>
<td>Name</td>
</tr>
<tr>
<td>----------</td>
<td>------</td>
</tr>
<tr>
<td>Pollen</td>
<td><em>Parietaria judaica</em> (Pellitory, wall)</td>
</tr>
<tr>
<td></td>
<td><em>Phragmites communis</em> (Reed)</td>
</tr>
<tr>
<td></td>
<td><em>Pinus radiata</em> (Pine)</td>
</tr>
<tr>
<td></td>
<td><em>Pinus strobus</em> (Pine, Eastern White)</td>
</tr>
<tr>
<td></td>
<td><em>Platanus acerifolia</em> (Plane tree) (^2)</td>
</tr>
<tr>
<td></td>
<td><em>Plantago lanceolata</em> (Plantin, English)</td>
</tr>
<tr>
<td></td>
<td><em>Tilia cordata</em> (Linden)</td>
</tr>
<tr>
<td></td>
<td><em>Poa pratensis</em> (Bluegrass, Kentucky)</td>
</tr>
<tr>
<td></td>
<td><em>Podocarpus spp.</em> (^1)</td>
</tr>
<tr>
<td></td>
<td><em>Populus deltoides</em> (Cottonwood eastern)</td>
</tr>
<tr>
<td></td>
<td><em>Populus nigra</em> (Poplar, black)</td>
</tr>
<tr>
<td></td>
<td><em>Populus trichocarpa</em> (Cottonwood, Black)</td>
</tr>
<tr>
<td></td>
<td><em>Quercus alba</em> (^2)</td>
</tr>
<tr>
<td></td>
<td><em>Quercus ilex</em> (Oak, live)</td>
</tr>
<tr>
<td></td>
<td><em>Quercus robur</em> (Oak, red)</td>
</tr>
<tr>
<td></td>
<td>Rice flour</td>
</tr>
<tr>
<td></td>
<td><em>Robinia pseudoacacia</em> (Acacia, false)</td>
</tr>
<tr>
<td></td>
<td><em>Rumex acetosella</em> (Sorrel)</td>
</tr>
<tr>
<td></td>
<td><em>Salsola kali</em> (Saltwalt, Russian thistle)</td>
</tr>
<tr>
<td></td>
<td><em>Sambucus nigra</em> (Elder, European)</td>
</tr>
<tr>
<td></td>
<td><em>Schnus molle</em> (pepper tree)</td>
</tr>
<tr>
<td></td>
<td><em>Solidago virga-aurea</em> (Golden rod)</td>
</tr>
<tr>
<td></td>
<td><em>Sorghum halepense</em> (Johnson grass) (^2)</td>
</tr>
<tr>
<td></td>
<td><em>Triticum sativum</em> (Wheat, cultivated)</td>
</tr>
<tr>
<td></td>
<td><em>Tamarix gallica</em> (^2)</td>
</tr>
<tr>
<td></td>
<td><em>Taraxacum officinale</em> (Dandelion)</td>
</tr>
<tr>
<td></td>
<td><em>Ulmus americana</em> (Elm, American)</td>
</tr>
<tr>
<td></td>
<td><em>Ulmus minor</em> (Elm, English)</td>
</tr>
<tr>
<td></td>
<td><em>Urtica dioica</em> (^2)</td>
</tr>
<tr>
<td></td>
<td>Wheat flour</td>
</tr>
<tr>
<td></td>
<td><em>Zae Mays</em> (^2)</td>
</tr>
<tr>
<td>Category</td>
<td>Name</td>
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<tr>
<td>------------</td>
<td>-------------------------------------------</td>
</tr>
<tr>
<td>Mite</td>
<td><em>Acarus siro</em></td>
</tr>
<tr>
<td></td>
<td><em>Alopecurus geniculatus</em></td>
</tr>
<tr>
<td></td>
<td><em>Blomia tropicalis</em></td>
</tr>
<tr>
<td></td>
<td><em>Dermatophagoides farinae</em></td>
</tr>
<tr>
<td></td>
<td><em>Dermatophagoides pteronyssinus</em></td>
</tr>
<tr>
<td></td>
<td><em>Glycophagus domesticus</em></td>
</tr>
<tr>
<td></td>
<td><em>Lepidoglyphus destructor</em></td>
</tr>
<tr>
<td></td>
<td><em>Suidasia medanensis</em></td>
</tr>
<tr>
<td></td>
<td><em>Tyrophagus putrescentiae</em></td>
</tr>
<tr>
<td>Epithelial</td>
<td>Budgerigar</td>
</tr>
<tr>
<td></td>
<td>Cat</td>
</tr>
<tr>
<td></td>
<td>Cow</td>
</tr>
<tr>
<td></td>
<td>Dog</td>
</tr>
<tr>
<td></td>
<td>Feathers mix(duck, chicken)</td>
</tr>
<tr>
<td></td>
<td>Goose</td>
</tr>
<tr>
<td></td>
<td>Goat</td>
</tr>
<tr>
<td></td>
<td>Guinea pig</td>
</tr>
<tr>
<td></td>
<td>Hamster</td>
</tr>
<tr>
<td></td>
<td>Horse</td>
</tr>
<tr>
<td>Others</td>
<td>Latex</td>
</tr>
<tr>
<td></td>
<td><em>Dicranopteris spp.</em></td>
</tr>
</tbody>
</table>

4.3.2.2 Procedure of Immunodotblot Array Analysis

Figure 34 shows the procedure of the immunoarray dot blot system. After protein extraction and standardization, allergen extractions were carefully positioned into a 384-well plate with a duplicate for every allergen. The 7.5cm x 11.5cm plate was divided evenly into three areas, about 7.5 x 3.5 cm² each. Each area was applied with the same pattern of allergen extractions listed in Table 53. A series of dilutions of standard human
serum IgE (National Institute for Biological Standards and Control, Herts, UK) with dilution ranging from 8 IU/ml to 125 IU/ml was used as a positive control. BSA and extraction buffer were used as the negative protein control and buffer controls, respectively. A pure nitrocellulose membrane (BioRAD, Richmond, CA) was selected as the support material. VP 386 MULTI-BLOT™ replicator (V&P Scientific, CA) with pins coated with a hydrophobic material, and a VP 382 MULTI-PRINT™ were used for solution transfer from well plate onto membrane. The replicator was dipped into the wells for about ten seconds before being applied onto the membrane. To ensure that a sufficient amount of proteins was attached onto the membrane, the transfer operation was repeated five times. Each time about 0.2 µl of allergen extraction was delivered by each pin, and finally about 1µg of protein could be dotted onto the membrane. The membrane was air dried at room temperature overnight. The following morning, the membrane was blocked in 3% (w/v) skimmed milk powder (Anlene™, New Zealand) in 1× PBS-T (Phosphate-buffered saline with 0.05% Tween 20 from Cayman Chemical Co. Ltd., pH 7.4) at room temperature for one hour. The membrane was then washed with PBS-T three times, for ten minutes each time. 150 µl serum was diluted in PBS with a ratio of 1:1. The membrane was incubated in the sera dilution overnight at 4°C. After washing by PBS-T with the above mentioned procedure, the membrane was then incubated with 300 µl alkaline phosphatase-conjugated goat anti-human monoclonal IgE (Sigma-Aldrich Inc., US) which had been diluted in PBS with a ratio of 1:1000. The incubation was carried out at room temperature for two hours, followed by washing with PBS-T. A NBT/BCIP color development substrate (Promega Co. Ltd.) was prepared in alkaline phosphatase buffer (100 mM Tris HCl pH 9.0, 150 mM NaCl, 1mM MgCl2). The membrane was
immersed into NBT/BCIP for color development for about 30 minutes. The membrane was then washed with distilled H₂O and scanned (HP Scanjet 6100C). A micro Image system (Micro-Image for Windows, version 4.0, Olympus Europe, Hamburg, Germany) was used to digitize the color intensity of each dot.

**Figure 34.** Procedure of Dot-Immunoarray system.

### 4.3.2.3 Solution Transfer Efficiency of Replicator

BSA with a concentration of 0.2 mg/ml was used for testing the transfer efficiency following the transfer procedure mentioned in 4.3.3.2, i.e., a dot brought five times onto the membrane with the replicator. The membrane was air dried at room temperature overnight and stained with amino black. Color intensity was scanned and digitized with the Micro Image system.
4.3.2.4. Effect of Washing Procedure

The effect of the washing procedure on the final amount of protein on the membrane was also evaluated by using the same amino black-imaging methods after washing with PBS-T and TBS-T with a series concentrations.

4.3.2.5 Validation of Immunoarray System by Self-developed ELISA

To validate the sensitivity and specificity of the immunoarray system, a self-developed ELISA test was done with randomly selected sera and allergens. A total of 24 allergen sources from different groups were tested. They were pollen (Acacia spp., Arecastrum romanzoffianum, Betula verrucosa, Casuarina equisetifolia, Cryptomeria japonica, Cynodon dactylon, Eucalyptus globulus, Elaeis guineensis, Olea europea, Pinus strobus, Quercus alba, Urtica dioica), fungi (Candida albicans, Curvularia lunata, Fusarium solani, Penicillium notatum, Schinus mollis and Stemphylium botryosum), mite (Suidasia medanensis), food of plant origin (soya bean, tofu), and food of animal origin (banana prawn, sea bream fish and selar fish). Extraction method was similar to that mentioned in 4.3.2.1. except that PBS was used instead of the extraction buffer. Allergen extractions (0.01 mg/ml) were coated in a 96-well plate (NUNC MaxiSorp, Denmark) at 4°C overnight. After blocking with 1% BSA, the diluted sera (1:1) were added for the first incubation. Biotinylated mouse anti-human IgE (BD PharMingen) was added for the second incubation, following by incubation with avidin-alkaline phosphatase (BD PharMingen). TMB (3,3’,5,5’-tetramethylbenzidine, Sigma-Aldrich) was used for the color development.
4.3.3 Determination of sIgE to *Trichophyton rubrum* by the ImmunoCAP System

The serum specific IgE (sIgE) to *Trichophyton rubrum* in the sera of nasal polyp and chronic sinusitis patients was tested using the ImmunoCAP system. Specific IgE ≥0.35 KU/L was regarded as atopy.

4.3.4 Determination of sIgE to *Trichophyton rubrum* by ELISA

A commercial ELISA kit of *Trichophyton rubrum* from RIDASCREEN® (R-Biopharm AG, Darmstadt, Germany) was used to test the sera specific IgE (sIgE) in nasal polyp and chronic sinusitis patients. In the first incubation, positive and negative controls and sera were applied into the wells and incubated at 37°C for one hour. Washing buffer was used to wash wells six times after the first incubation. In the second incubation, 50 µl of RIDASCREEN® AllergieESpezifisch (anit-human-IgE conjugate) was added into each well, covered and incubated at 37°C for one hour. The wells were washed six times after incubation, following incubation with 100 µl of the ready-to-use substrate solution in each well for 15 minutes at 37°C in the dark. 50 µl of RADIOSCREEN® AllergieStopp was added in each cavity in order to stop color development. At 405 nm against a wavelength of 620 nm, the measurement of the optical density was performed.

4.3.5 Statistical Analysis

The data analysis was done with the program of SAS 8.02. ANOVA was used for the analysis of the transfer efficiency and for the evaluation of washing effects of the immunoarray system. Two standard deviations (SD) above the negative reactions for each allergen source tested were used as the cut off points for positive results. The
concordances were tabulated based on positive and negative reactions.

4.4 Results

4.4.1 Optimization of Supporting Media

The nitrocellulose membrane, PVDF membrane were optimized for retaining the loaded protein. Protein concentrations on the PVDF membrane (170.83 ± 8.93 OD) were significantly higher (p<0.0001) compared to that on the nitrocellulose (124.08 ± 7.92 OD) membrane. However, the background obtained using the PVDF membrane (66.42 ± 15.56 OD) was found to be higher (p<0.0001) than that of the nitrocellulose membrane (27.85 ± 9.44 OD). Pre-wetting with methanol before usage of the PVDF membrane reduces its feasibility as well. The nitrocellulose membrane was chosen as the supporting media in our immunodot array system.

4.4.2 Evaluation of Transfer Efficiency

![Optical density readings of the protein dots (BSA) at different concentrations. Maximum, minimum, means and SD (error bar) of dots are shown.](image)

Figure 35. Optical density readings of the protein dots (BSA) at different concentrations. Maximum, minimum, means and SD (error bar) of dots are shown..
Figure 35 shows the transfer efficiency test with a series of BSA concentrations, i.e., 0.0625 mg/ml to 1 mg/ml. The amount of protein attached onto the membrane increased with higher BSA concentrations.

4.4.3 Optimization of Washing Buffer

A variety of washing buffers were optimized, i.e., PBS-T and TBS-T with a series concentrations. An increased concentration of Tween-20 significantly decreased the protein loading onto the membrane (Figure 36). On the other hand, a decreased concentration of Tween-20 may result in a higher background due to the lower washing effects on non-specific binding. 0.05% PBS-T was chosen as the washing buffer due to its proper washing efficiency and protein retaining capacity.

![Figure 36](image)

**Figure 36.** Effects of different concentrations of Tween 20 detergent in PBS washing buffer. Means and SD (error bars) are shown.
4.4.4 Validation of the Immunoarray System

The self-developed ELISA was carried out in selected sera with selected allergens. The positive/negative concordance was calculated and compared with the result from the immunoarray system. Result is shown in Table 54.

Table 54. Concordance between the immunoarray system and the self-developed ELISA of randomly selected allergens.

<table>
<thead>
<tr>
<th>Allergen</th>
<th>Concordance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>++</td>
</tr>
<tr>
<td><strong>Olea europaea</strong> (Olive)</td>
<td>94.4</td>
</tr>
<tr>
<td>Banana prawn</td>
<td>91.7</td>
</tr>
<tr>
<td>Soya bean</td>
<td>91.7</td>
</tr>
<tr>
<td><em>Quercus alba</em></td>
<td>91.3</td>
</tr>
<tr>
<td>Tofu</td>
<td>90.9</td>
</tr>
<tr>
<td><strong>Pinus strobus</strong></td>
<td>90.0</td>
</tr>
<tr>
<td><em>Fusarium solani</em></td>
<td>84.2</td>
</tr>
<tr>
<td>Selar fish</td>
<td>83.3</td>
</tr>
<tr>
<td>Sea bream fish</td>
<td>81.8</td>
</tr>
<tr>
<td><em>Betula verrucosa</em></td>
<td>78.9</td>
</tr>
<tr>
<td><em>Curvularia lunata</em></td>
<td>75.0</td>
</tr>
<tr>
<td><strong>Elaeis guineensis</strong></td>
<td>39.1</td>
</tr>
<tr>
<td><em>Acacia spp.</em></td>
<td>72.2</td>
</tr>
<tr>
<td><em>Schinus mollis</em></td>
<td>72.2</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>70.6</td>
</tr>
<tr>
<td><em>Cynodon dactylon</em></td>
<td>38.9</td>
</tr>
<tr>
<td><em>Suidasia medanensis</em></td>
<td>66.7</td>
</tr>
<tr>
<td><em>Stemphylium botryosum</em></td>
<td>55.6</td>
</tr>
<tr>
<td><em>Penicillium notatum</em></td>
<td>64.7</td>
</tr>
<tr>
<td><em>Casurina equisetifolia</em></td>
<td>60.9</td>
</tr>
<tr>
<td><em>Cryptomeria japonica</em></td>
<td>56.5</td>
</tr>
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<td><em>Eucalyptus globulus</em></td>
<td>10.5</td>
</tr>
<tr>
<td><em>Urtica dioica</em></td>
<td>55</td>
</tr>
<tr>
<td><em>Arecastrum romanzoffianum</em></td>
<td>72.7</td>
</tr>
</tbody>
</table>

1, hyphen (-), no sera tested.
Figure 37 (I to V) shows some ROC curves (Receiver Operating Characteristic curve) of selected allergens tested by the immunodot blot array system as compared to the evaluations by self-developed ELISA. Accuracy is measured by the area under the ROC curve. An area of 0.9 to 1 represents an excellent test; an area of 0.8 to 0.9 represents a good test; a fair test is represented by an area of 0.7 to 0.8; while an area of 0.6 to 0.7 represents a poor test; an area of 0.5 to 0.6 represents a failed test; areas below 0.5 indicates a worthless test.

**I. ROC Curve of Elaeis guineensis (area under the curve 0.795)**

![ROC Curve](image)

**Figure 37 (I to V).** ROC curves (Receiver Operating Characteristic curve) of selected allergens tested by the immunodot blot array system as compared to the evaluations by self-developed ELISA.
Figure 37, Continued.

II. ROC Curve of Fusarium solani (area under the curve 0.844)

III. ROC Curve of Betula verrucosa (area under the curve 0.767)
Figure 37, Continued.

IV. ROC Curve of Candida albicans (area under the curve 1.0)

V. ROC Curve of Stemphylium botryosum (area under the curve 0.70)
4.4.5 Common Allergens Identified in the Study Groups

Table 55 shows the common allergens (top 20) identified in our study groups. The allergic rhinitis patients were prone to react to multiple allergens, as compared with other groups. Although dust mite, fungus, pollen and food allergens could all be identified in patients with different diagnosis, the species patterns seemed quite variable in different study groups. In addition, nasal polyp and chronic sinusitis patients had similar patterns of specific IgE. In both nasal polyp and chronic sinusitis patients, serum specific IgE to *Trichophyton rubrum* was identified with surprisingly high incidence rate, which was 89% and 86%, respectively.

4.4.6 Quantified sIgE to *Trichophyton rubrum* in Nasal Polyp and Chronic Sinusitis Patients via ImmunoCAP

ImmunoCAP of serum sIgE (specific IgE) to *Trichophyton rubrum* in nasal polyp and chronic sinusitis patients all proved to be negative. There was totally no concordance between the results of the immunoassay system and the ImmunoCAP system.

4.4.7 Quantified sIgE to *Trichophyton rubrum* in Nasal Polyp and Chronic Sinusitis Patients via ELISA

Of the 17 chronic sinusitis/nasal polyp patients identified with sIgE to *Trichophyton rubrum* in the sera, only three were proved to be positive by the ELISA kit with a weak reaction.
<table>
<thead>
<tr>
<th>Category</th>
<th>Nasal Polyps</th>
<th>Chronic Sinusitis</th>
<th>Allergic Rhinitis</th>
<th>Non-allergic Rhinitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dust Mite</td>
<td><em>Dermatophagoidespteronyssinus</em></td>
<td><em>Tyrophagus putrescentia</em></td>
<td><em>Alopecurus geniculatus</em></td>
<td><em>Glycophagus domesticus</em></td>
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<tr>
<td></td>
<td><em>Tyrophagus putrescentia</em></td>
<td><em>Suidasia medanensis</em></td>
<td><em>Blomia tropicalis</em></td>
<td><em>Dermatophagoides farinae</em></td>
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<tr>
<td></td>
<td><em>Suidasia medanensis</em></td>
<td></td>
<td><em>Glycophagus domesticus</em></td>
<td><em>Blomia tropicalis</em></td>
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<td><em>Glycophagus domesticus</em></td>
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<td><em>Dermatophagoides farinae</em></td>
<td><em>Alopecurus geniculatus</em></td>
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<td></td>
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4.5 Discussion

RAST is commonly used as the in vitro test for the diagnosis of allergy. This technique requires large amounts of serum (300 µl per test) and reagents. Although the diagnosis of allergy is based on both allergy tests and patients’ history, the limitation of commercial allergens may lead to underestimation of allergic disease. In our study patients with nasal polyp/chronic sinusitis, although eosinophil numbers, which are closely related with allergy, remarkably increased in the tissues, the ImmunoCAP tests did not indicate a higher incidence rate of atopy in these patients than the general population (chapter 2). Because of the limitation of the ImmunoCAP test, it is possible that specific IgE to other uncommon allergens may exist. To look further into the role of allergy in nasal polyps and chronic sinusitis, we were able to develop a dot-immunoassay system containing a large panel of allergens. The procedure of the test is simple. Only 150 µl serum is needed for the test of specific IgE to 185 common and uncommon allergens. In addition, the use of heterogeneous protein extractions may provide a more proper diagnosis of allergen exposure in the natural environment, as compared to purified or recombinant allergens. There was also good concordance between the immunoarray system and the self-developed ELISA.

Although there was correlation between the intensity of protein attached onto the membrane and the concentration of protein solutions, the validation of transfer efficiency by BSA showed a large range of proteins on the membrane. This may be due to an unbalanced transfer volume of the pin-type replicator. The properties of allergens may also alter the character of solutions, and hence lead to a larger discrepancy for solution
transfer. Other important factors contributing to the differences on the volume transferred onto the membrane include variation in speed on removing the replicator, stock well volume and dwell time of the pin on membrane (datasheet from V&P Scientific, California). These factors may limit the quantitative aspect of the analysis of the test. A more efficient and standardized transfer system may increase the reproducibility and precision of the method, even improve the test to a quantitative assay of specific IgE.

Based on the result of the immunoarray system, we were able to identify a large panel of specific IgE in the study groups. Allergic rhinitis patients were more prone to be reactive to multi allergens. Patients with a different diagnosis may have unique allergen patterns. Nasal polyp and chronic sinusitis patients had a good deal of allergens in common. For example, Trichophyton spp. is the most commonly identified fungus allergen in nasal polyps and chronic sinusitis patients, whereas in allergic and non-allergic patients, that is not the case. As allergy is a reaction between allergen and host, the variation of allergen patterns in patients with different diagnosis may reflect not only the properties of the allergens, but also the contributions of the various predisposition factors of the host.

The dot-immunoarray system is newly developed and needs greater standardization. Trichophyton rubrum, an important fungus identified both in nasal polyp and chronic sinusitis patients, is not reported as an important allergen in these diseases in the literature. The validation by the traditional ImmunoCAP system and commercial ELISA kit did not support our finding of the importance of Trichophyton rubrum. The lack of concordance of the tests may be due to a false positive outcome of our immunoarray
system. The use of crude extract, purified or recombinant allergens could be a possible factor also for different reactions. In addition, standardization of allergen extracts may induce large discrepancies in the results. So far, there are very few commercial standardized allergens. For example, only 19 licensed standardized allergen extracts are available in the US and most of them are mites and pollens but not fungus. Although commercial fungal allergens are available from a few companies, it has been suggested that there may be a large variation of quality and quantity according to their culture conditions and strains.\textsuperscript{13}

In conclusion, the diagnosis of atopy may be underestimated due to the limitation of commercial allergens. Our study of the immunoarraydot blot system may provide a promising method in the diagnosis of atopy. However, it needs far more standardization. Results of our test showed different allergen patterns in patients with different diagnosis, suggesting the contribution of host factors in addition to the properties of allergens in an allergic reaction.
Reference List

Chapter 5. The Involvement of *Trichophyton rubrum* in the Pathogenesis of Nasal Polyps and Chronic Sinusitis

5.1 Role of Fungi in the Pathogenesis of Nasal Polyps and Chronic Sinusitis

5.1.1 Incidence of Allergic Fungal Sinusitis (AFS) in Nasal Polyps and Chronic Sinusitis

Fungi are important outdoor and indoor allergens. Fungal species derived from Ascomycota (*Aspergillus famigatus, Cladosporium herbarum, Penicillium chrysogenum, Alternaria alternata, Trichophyton rubrum, Candida albicans*) and the Basidiomycota (*Malassezia furfur, Psilocybe cubensis, Coprinus comatus*) are considered to have the utmost clinical importance. These fungi may release allergens in the form of spores as well as mycelia and yeast forms and initiate immediate or delayed-type hypersensitivity in the host.

The role of fungi in the pathogenesis of nasal polyp and chronic sinusitis still remains controversial. Fungal sinusitis can be classified into four primary categories: acute/fulminant (invasive), chronic/indolent (invasive), fungus ball and allergic fungal sinusitis (AFS), which were introduced in chapter 1.3. AFS is a chronic, noninvasive fungal sinusitis which is distinct from the other three types. About 2% to 10% of the patients with chronic sinusitis who required sinus surgery have AFS. Patients with AFS usually have nasal polyps, with percentages varying from 75% to 100%. The most important fungi related with nasal polyps and chronic sinusitis are *Aspergillus spp., Candida spp., Alternaria spp., Bipolaris spp., Curvularia spp., Drechsler spp.*
Exserohilum spp., Rhizopus spp., Fusarium spp. and Chrysosporium spp.. Exserohilum spp., Rhizopus spp., Fusarium spp. and Chrysosporium spp.. Positive fungus cultures of mucosa, mucin or secretion in the nasal/sinus cavity from nasal polyp or chronic sinusitis patients ranges from 7% to 93%. However, it was reported that there was no difference between the percentage of fungus identified in patients with chronic sinusitis/nasal polyps and normal controls. In 1999, Ponikau et al. evaluated the criteria in 210 patients with chronic sinusitis with/without nasal polyps. 93% of them were diagnosed as AFS based on histology and fungal culture. 101 patients were tested surgically. The fungal culture was positive in 96% of the 101 patients whereas 97% of them had allergic mucin. An interesting finding was that 100% of the healthy volunteers had a positive fungal culture as well. Braun et al. reported similar results with an incidence rate of 91.3% of positive culture in both chronic sinusitis patients and healthy controls. In another study of fungus detection by PCR, fungus DNA was evidenced in the nasal cavities of of 42% chronic sinusitis patients and 40% of normal controls. In another study by Lebowitz et al., the reported incidence rate of the fungus positive culture was 55.6% out of 45 patients with chronic sinusitis. The most commonly identified ones were Aspergillus (52%) and Penicillium (32%). Coexistence of multi fungi was found in 20% in the patients with positive culture. In other studies, no positive fungal culture from controls was reported. Laboratory methods may be the root of large differences in the outcomes of different studies. The variety of reactions to fungus suggests the importance of the host factor in addition to the environment and the properties of fungal pathogens. It was proposed that the immune reaction in AFS is affected by
exposure to fungi, the Ig-E mediated allergy, the specific T cell HLA receptor, as well as the condition of the local mucosal defense system. Other local conditions, such as deviated septum, massive nasal polyps and obstructed ostiomeatal complex, which result in bad ventilation, may contribute to the progress of AFS and lead to prolonged fungus contact.

5.1.2 Mechanism of AFS, IgE-Mediated Allergy?
The mechanism underlying AFS is far from clear. The term ‘allergic fungal sinusitis’ was adopted mainly because of two reasons: the presence of allergic mucin; and the infiltration of eosinophils in the nasal/sinus mucosa. However, the role of IgE-mediated allergy in AFS is subject to debate. Supportive evidence is mainly found in the major involvement of eosinophils rather than neutrophils, and the presence of specific IgE to fungus by skin prick test, RAST or ELISA. It was also suggested that serum total IgE>1000 IU/ml is a good predictor for AFS. Other evidence includes common presence of AFS in immunocompromised young patients with atopy, lack of invasion and clinical progression to infection. Controversial reports questioned the role of IgE-mediated allergy in AFS. It was reported that although fungus is frequently identified in nasal polyp and chronic sinusitis patients, IgE-mediated allergy was not identified in most of the patients. Therefore, it was suggested that ‘allergic fungal sinusitis’ should be replaced by ‘eosinophil fungal sinusitis’ because an non-IgE mediated pathway may be responsible for the eosinophil infiltration.
In addition to IgE-mediated hypersensitivity, type III hypersensitivity (immune complex reaction) mediated by fungus specific IgG (slgG) has been shown.\textsuperscript{14} An IgG mediated type II hypersensitivity (cytotoxic reaction) was also proposed in AFS.\textsuperscript{18} However, it was reported that fungus slgG was identified in both AFS patients and non-AFS patients, including non-AFS polypoid chronic rhinosinusitis (CRS) patients, allergic rhinitis and normal controls. Although slgG in the non-AFS polyp CRS group was not as high as that in AFS patients, it was significantly higher than in allergic rhinitis and controls. In addition, an increase in the fungal slgG was associated with the severity of nasal polyp disease. The presence of slgG may reflect the host’s defensive response.\textsuperscript{15}

Another major theory suggested infection played a role in the pathogenesis of AFS. Although \textit{Staphylococcus aureus} (\textit{S. aureus}) is commonly cultured from the sinus mucosa of AFS patients, the treatment by antistaphylococcal antibiotics is not helpful.\textsuperscript{19} Further evidence shows that clinical presentation, surgical sinus histopathology and immunologic findings are not consistent with bacterial infection.\textsuperscript{20} Infection does not seem to be the primary etiology of AFS.

\subsection*{5.1.3 Superantigen, a Rising Theory in the Pathogenesis of Nasal Polyps and Chronic Sinusitis}

An antigen is substance which can bind a specific antibody or T cell receptor. There are varieties of antigen receptors on B and T lymphocytes. These antigen receptors are
essential for the maturation and differentiation of the lymphocytes. Surface Ig-α and Ig-β are antigen receptors of B cells. On the surface of most T cells, TCR (T cell receptor) consists of α and β heterodimers, whereas as some other T cells bear γδ chains. Antigens are processed by APCs (antigen presenting cells, e.g., dendritic cells, mononuclear phagocytes and B lymphocytes). APCs are necessary to form MHC-associated peptides. The interaction of naïve T cells and the processed antigens will lead to activation, proliferation and differentiation of T cells into effector and memory cells. CD4+ (CD3+, CD4+, CD8-) T cells recognize class II MHC (major histocompatibility complex) molecules, whereas CD8+ (CD3+, CD4-, CD8+) T cells recognize class I MHC molecules. Another subset of lymphocytes, natural killer cell, lyses its target cell without MHC restriction.

Superantigens are antigens which are not processed by APCs but cross link class II and Vβ independently of any direct interaction between MHC and TCR molecules. They are potent mitogens which stimulate whole lymphocyte subpopulations sharing the same TCR V β or Ig V_H family, independently of antigen specificity. It was hypothesized that genetic defects on TCR V β chain results in immunopathology which affects 16% of the population.¹⁸ Superantigen is able to bind with many TCR V β gene motifs. Normally, an antigen will activate <0.01% of the body’s total amounts of T cells, whereas a superantigen will result in activation of up to 30% of the total amount of T cells.¹⁸ This activation will induce systematic toxicity. Many systematic diseases are proposed to be associated with superantigens, such as human retro virus
in breast cancer, Type I diabetes and multiple sclerosis. Known microbes with superantigen activity include *S. aureus*, retro virus, Epstein-Barr virus and fungus, such as *Alternaria* and *Bipolaris specifera*.

AFS was recently suggested as a superantigen-induced disease. In AFS patients, nasal mucous contains eosinophils, Charcot-Leyden crystals, sIgG but lacks T helper cells and APCs. Fungal antigens in the peripheral blood are able to activate T helper cells and recruit eosinophils through the release of cytokines. However, lymphocytes from normal controls do not show eosinophil recruitment induced by fungal antigens. Therefore, the role of superantigens in AFS was suggested because of the bypass of APCs. Further evidence of fungus superantigens is that patients with ABPA (Allergic Bronchopulmonary Aspergillosis) possess TCR V\(\beta\)13, whereas patients with hypersensitivity to *A. familatus* but no ABPA have TCR V\(\beta\)1. TCR V\(\beta\)13 is a common microbial TCR superantigen-binding motif but TCR V\(\beta\)1 is not. It was proposed that Asp f1 and Asp f4 may possess the ability of a superantigen. *Alternaria spp.* which belongs to dematiaceous fungal group, and is commonly found in AFS, is proposed to act as a superantigen as well. However, the binding of the superantigen with TCR V\(\beta\) motifs may be the necessary but not sufficient factor to induce correlated diseases. For example, as we mentioned, a superantigen binds with TCR and HLA class II molecules simultaneously. HLA class II molecules show preferential binding for certain antigens. Schubert et al. proposed a consequential development of a superantigen related disease. The initial step is decided by the following factors:
exposure to microbes; mucosal factors contributing to the colonization and persistence of the microbes; and microbial binding proteins of epithelial cells of the host. Genetic factors related with TCR Vβ and HLA class II molecules are essential for host reaction. In some cases, co-presence of other superantigens such as SEB may be necessary for the reaction. Preexistence of various pathological conditions, such as type-I mediated allergy, Th1/Th2 cytokine dysregulation, leukotriene dysregulation will amplify the T cell response induced by the superantigen to form systemic disorders characterized by eosinophilic-lymphocytic chronic inflammation with varying intensity and chronicity.

The role of superantigens in the pathogenesis of nasal polyps and chronic sinusitis has been raised. The commonly reported superantigen in nasal polyps and chronic sinusitis is from *S. aureus*, including *Staphylococcus* enterotoxin A (SEA), *Staphylococcus* enterotoxin B (SEB), and toxic shock syndrome toxin 1 (TSST-1). SEB induces bronchial hyperresponsiveness and the typical histophathological findings in asthma. SEB was also proved to stimulate cysteinyl leukotrienes in vivo. TSST-1 is able to augment an antigen specific IgE response in atopic patients in vitro. From the study of Ponikau et al., most patients (93%) with chronic sinusitis with/without nasal polyps can be diagnosed as AFS. Evidence for fungal superantigens in the pathogenesis of nasal polyps and chronic sinusitis has been sought. In CHS/NP patients, specific IgE to SEB and fungus always coexist, suggesting that the TCR Vβ motifs are similar to both of them. In addition, chronic
sinusitis/nasal polyp and systemic fungus symptoms were closely correlated with fungus exposure. All the symptoms improved when fungal exposure was removed or blocked by antimicrobial nose sprays, but the symptoms returned when the treatment stopped.\textsuperscript{18}

In summary, the role of fungi in the pathogenesis of nasal polyps and chronic sinusitis may be far underestimated due to limitation and standardization of detection methods. In addition, the role of superantigens, especially fungal superantigens identified in recent years is challenging the traditional theories of fungi related diseases. However, evidence is still lacking. Most proposed mechanisms have not been investigated yet.

5.2 Epidemiology and Biology of \textit{Trichophyton rubrum}

5.2.1 Epidemiology of \textit{Trichophyton rubrum}

\textit{Trichophyton rubrum} (\textit{T. rubrum}) is a filamentous fungus which was first described in 1911. Southeast Asia was speculated as the original endemic area of \textit{T. rubrum}, which spread worldwide after the world war II.\textsuperscript{26} \textit{T. rubrum} belongs to the dermatophytes which were reported as the most common isolated pathogen in cutaneous fungal infection, as compared with yeast and nondermatophyte molds.\textsuperscript{27,28} The prevalence of dermatophytosis in the general population was reported to be 13.5\%.\textsuperscript{29} It was estimated that 30-70\% of the adults were asymptomatic carriers of \textit{Trichophyton spp.}\textsuperscript{30} Most of the surveys carried out in the last few decades, including the US, Europe, Africa and Australia, reported that \textit{T. rubrum} is the dermatophyte species with
the highest incidence of isolation which is up to 93%.27,31-39 Studies in various regions in Asia, including China, India, Thailand, Malaysia, Korea and Vietnam, reported similar findings.40-48 Other important fungal pathogens in cutaneous fungal infection include Trichophyton mentagrophytes,49 Trichophyton violaceum,50 Candida albicans,51 Microsporum audouinii,52 Microsporum canis53 and Epidermophyton floccosum.52

5.2.2 Trichophyton rubrum Related Diseases

T. rubrum is closely associated with skin and nail infection, i.e. onychomycosis, tinea corporis, tinea cruris, tinea manuum, tinea pedis, tinea unguium and tinea barbae.27,54,55 These diseases correlated with T. rubrum are common in both children and adults.35,56,57 The incidence of infection may increase with age.35,58 Other causally related diseases are lesion of the sole,59 kerion celsi,60 and pustular lesions,61 Cushing’s syndrome, lymphoblastoma, connective tissue disease and urticaria.62 Interestingly, a study reported that T. rubrum was isolated from 29 out of 32 patients with auditory meatus infection.63 There are many factors affecting the risk of infection to T. rubrum. In patients with immnosupressive conditions, for example, HIV, autoimmune disease and use of immnosuppressive drugs after transplantation, the incidence of T. rubrum infection is very high and sometimes it causes deep infections.64-68 Patients with diabetes or peripheral vascular disease also show the infection more frequently.69,70 Other factors such as hormone level, environment, lifestyle have also been found relevant reported.70-72 T. rubrum infection has a high
recurrence rate between 33% and 60%, and it is often found in those persons with fungus infection resistant to treatment.\textsuperscript{73,74} It was suggested that the \textit{T. rubrum} infection may be a familial disease related with autosomal inheritance.\textsuperscript{75-77}

The association between \textit{T. rubrum} and atopy has been suggested. \textit{T. rubrum} infection in atopic diseases, i.e., atopic dermatitis, asthma and allergic rhinitis has been widely reported.\textsuperscript{78-84} These diseases often occur systematically. Atopic dermatitis is suggested to be the initial step, giving rise to asthma and allergic rhinitis.\textsuperscript{85} Asthma related with \textit{T. rubrum} infection is often perennial or intrinsic and usually associated with nail or skin infections.\textsuperscript{82,86,87} These patients have a marked eosinophilia, commonly coexisting with rhinosinusitis, and negative allergy test to common allergens. The treatment of a superficial fungus infection improved the symptoms of asthma. It was suggested that \textit{T. rubrum} may be inhaled with nail dust or absorbed systematically. Further evidence by means of skin prick test and airway challenge in patients with chronic asthma or rhinitis, led to an estimated incidence rate of immediate bronchial and nasal hypersensitivity to \textit{Candida albicans}, \textit{Trichophyton} and \textit{Epidermophyton} at around 8% to 10\%.\textsuperscript{88} Kaaman et al.\textsuperscript{89} suggested that the development of chronic dermatitis was exclusively related with \textit{T. rubrum}, but not other dermatophytes such as \textit{Trichophyton mentagrophytes} or \textit{Epidermophyton floccosum}. In addition, atopic respiratory disease was a more important predisposing factor than atopic eczema.

Although the association of atopy has been suggested by many studies, Escalante et
al.\textsuperscript{90} questioned it based on the equal incidence of culture proved chronic trichophytosis in atopic and nonatopic patients. Meanwhile, atopic diseases were not more common in patients with chronic trichophytosis. Whether a family history in addition to a positive allergy test is necessary for the diagnosis of atopy could contribute to the observed discrepancy. For example, Svejgaard et al.\textsuperscript{91} reported that although the incidence rate of atopy was as high as 70\% in their study patients with chronic \textit{T. rubrum} infection, there was little evidence of genetic predisposition.

In nasal polyp and chronic sinusitis patients, some studies with a large panel of allergens have mentioned the contribution of \textit{T. rubrum}. However, compared to other fungi, the incidence rate of \textit{T. rubrum} was much lower. For example, in the study of Ponikau et al.\textsuperscript{5} of chronic sinusitis patients with/without nasal polyps, fungi with relatively higher culture rate was \textit{Alternaria} \textit{spp.} (44.3\%), \textit{Penicillium} \textit{spp.} (43.3\%), \textit{Cladosporium} \textit{spp.} (39\%), \textit{Aspergillus} \textit{spp.} (29.5\%), \textit{Candida} \textit{spp.} (21.4\%), and \textit{Fusarium} \textit{spp.} (16.2\%). \textit{Trichophyton} \textit{spp.} was only cultured from 1\% of the samples. In another study by Asero et al.,\textsuperscript{8} the incidence of a positive skin prick test (SPT) in nasal polyp and chronic sinusitis patients to a large panel of allergens was investigated, including pollen, dust mite, animal dander and 13 mold allergens. 43\% of the nasal polyp patients were allergic to \textit{Candida albicans}, whereas less than 2\% of them were allergic to other molds, including \textit{Trichophyton}. The incidence rate in chronic sinusitis patients was even lower. From these data, \textit{T. rubrum} does not seem to be an important pathogen in the pathogenesis of nasal polyps and chronic sinusitis.
5.2.3 Role of *Trichophyton rubrum* in Related Diseases

5.2.3.1 Adhesion and penetration

Proteinases with the functions of degrading azocoll, collagen, laminin, fibronectin, azoalbumin, azocasein have been identified from *T. rubrum*.92-94 The affinity between conidia of *T. rubrum* and human keratinocytes was proved.95 *T. rubrum* has high keratinolytic activity as well as proteolytic activity which will lead to effective degradation of nail clipping and keratin.96-98 In addition, keratin is used by dermatophyte as a nutrient source.99 These characteristics may favor the erosion of skin and nail, following penetration of pathogens. Studies also identified carbohydrate-specific adhesins expression on the microconidia surface of *T. rubrum*, through which *T. rubrum* can recognize mannose and galactose to invade mammalian cells.100

5.3.2.2 Immediate hypersensitivity (IH) and delayed type hypersensitivity (DTH)

Infection of *T. rubrum* can elicit both strong humoral and cell-mediated immune responses in different individuals.101 The responses are evaluated in a skin test by immediate hypersensitivity (IH) and delayed type hypersensitivity (DTH). IH is related with allergy and mediated by IgE. DTH is cell-immunity with activation of macrophages by INF-γ produced by T lymphocytes and natural killer cells. Patients with chronic infection of *T. rubrum* mainly show IH with defect of DTH.102 Patients with acute *T. rubrum* infections mainly show DTH which reflects the host’s cell-immunity to eradicate pathogens as well as tissue damage. DTH was also identified in
30% of normal uninfected patients, suggesting a prior self-limited dermatophytosis.\textsuperscript{99} IH is correlated with RAST, IgE and IgG4 antibodies, whereas there is no such correlation found in DTH.\textsuperscript{103} Total IgE was often within the normal range in patients with IH.\textsuperscript{104} T cell lines for \textit{Trichophyton} antigens of DTH and IH subjects showed a distinctive cytokine production of Th1 (INF-\textgamma) and Th2/Th0 (IL-4, IL-5), respectively.\textsuperscript{105} Thus, a chronic infection by \textit{T. rubrum} will drive the Th1/Th2 balance to Th2 dominant immunity.\textsuperscript{106-108}

Studies of cell-mediated immunity in hosts with chronic \textit{T. rubrum} infections remain controversial. Sorensen et al.\textsuperscript{102} reported a decreased cell-immunity in the hosts with chronic \textit{Trichophyton} infections. The defective cell-immunity was not only limited to dermatophyte but also to other antigens. This may happen in patients with immunodeficient diseases. However, many other studies reported the defects in DTH was restricted only to \textit{T. rubrum}, while cell-immunity to other allergens, such as \textit{Trichophyton mentagrophytes} was normal.\textsuperscript{109,110} It was suggested that cell-immunity, as evaluated by DTH could more readily detected in patients with inflammatory infection.\textsuperscript{111} \textit{T. rubrum}, which belongs to anthropophilic species, often causes persistent infection with no obvious inflammatory response. The defective DTH evaluated by an in vitro lymphocyte transformation test showed that cell immunity was normal in patients.\textsuperscript{91,110,112,113} However, the discrepancy between in vivo and in vitro tests was restricted to \textit{T. rubrum}, without affecting other antigens, such as \textit{Trichophyton mentagrophytes} or \textit{Epidermophyton floccosum}.\textsuperscript{110} Multiple factors have
been suggested to explain this phenomenon. It was reported that *T. rubrum* can directly inhibit lymphocyte proliferation to recall antigens.\textsuperscript{112} Although it was originally thought that lymphocytes rather than APCs were the primary target of the inhibition,\textsuperscript{112} it is now widely accepted that the mannan component of the *T. rubrum* cell wall (TRM) can inhibit cell-immunity through binding with monocytes.\textsuperscript{114} Mannan from *T. rubrum* is a more potent immunosuppressor compared to that from other dermatophytes.\textsuperscript{115} It plays an important role in inhibiting antigen processing and presentation. It was further shown that TRM can affect the function of the epidermal tissue by binding with epidermal keratinocytes followed by internalization and catabolization.\textsuperscript{116} System factors, such as atopy, suppressor T cell, serum inhibitory factors or lack of stimulating serum factors have also been proposed.\textsuperscript{99,117} In addition, histamine which is released in IH may depress function of T cells.\textsuperscript{118}

Although the mechanism underlying the suppressed cell-immunity is not clear, studies have pointed towards suppressed peripheral lymphocytes and IgM in patients with chronic *T. rubrum* infection, but there was no correlation between the suppression and severity, duration or extent of the disease.\textsuperscript{119} Besides immuno disturbance, which preexists in some patients, unique response triggered by *T. rubrum* was identified. *T. rubrum* stimulated T lymphocytes always produce a suppressor cell clone which even suppresses itself.\textsuperscript{115} Maleszka et al.\textsuperscript{120} evaluated lymphocyte subsets in sera from patients with dermatophyte onychomycosis and controls. 22 out of the 35 patients were infected with *T. rubrum* while others were infected by *Trichophyton*
mentagrophytes, Trichophyton tonsurans or Epidermophyton floccosum. In the group of patients, lower levels of CD3+ T cells (pan T cells), CD4+ T cells (T helper cells) and CD3+HLA-DR+ cells (activated T cells) were identified before and after treatment compared to the controls. The level of CD8+ T cells (cytotoxic/suppressor T cells) in patients were slightly lower than controls before treatment. After treatment, CD8+ T cells increased in numbers that were slightly higher than controls. On the contrary, there was a higher percentage of natural killer cells in patients before treatment, but it decreased to a similar level as that in controls after treatment. There was no difference in B cell levels in the two groups before treatment, but after treatment, relatively lower B cell levels in patients than controls were identified. Lymphocyte levels were further compared between fully recovered patients and those patients for whom the treatment failed. The former group had relatively higher pan T cell, B cell and activated T cell levels, whereas the latter group showed a higher percentage of NK cells and T helper cells. It was clear that T cell activation was suppressed in the patients and this may also correlate with the severity and duration of the infection. The study of Petrini et al. demonstrated that chronic dermatophytosis patients had a decreased proportion of T cells with Fc receptors for IgM but increased T cells with IgG receptor. This is in agreement with increased level of IgG but not IgM in patients with a chronic infection of T. rubrum.\

5.3.2.3 Innate immunity

Besides the role of adaptive immunity, innate immunity is considered to play an
important role against *T. rubrum* infection through the role of PMNL (Polymorphonuclear leukocytes, i.e., eosinophils, neutrophils and basophils), the monocytes/macrophages, and natural killer (NK) cells. The role of PMNL against fungus infection was first evidenced in the study of *Aspergillus famigatus* and *Candida albicans*. *T. rubrum* was then shown to be a potent complement system activator as well. Plasma incubated with *T. rubrum* initiated C3 conversion and PMNL aggregation. *T. rubrum* may activate the complement system by its trypsin-like enzyme through the alternative pathway. This reaction may not only contribute to the host’s protection against *T. rubrum* but also to the development of inflammation. In addition, activation of C5a may produce neutrophil chemotactic factor (NCF) to induce neutrophil accumulation. The production of NCF will mimic the activity of the complement system. Further study proved that the activation of complement system may not able to kill *T. rubrum* but the burst of PMNL that follows may kill the pathogen by generating cytotoxic oxidative products. This oxidative system was proposed to be mainly executed by neutrophils, and to a less extent, by another phagocytic cell, the monocyte. Although both of the cells can ingest and destroy *T. rubrum* intracellularly, the extracellular killing mediated by oxidative products may play a more important role because of the difficulty to phagocytose fungus hyphae.

Monocytes bind selectively and specifically with the mannan component of *T. rubrum* cell wall (TRM) through mannan receptors. The binding will inhibit antigen processing and presentation. On the other hand, monocyte/macrophages can kill *T.
rubrum through phagocytosis and/or oxidative products. However, this effect may not be comparable to that of neutrophils. T. rubrum may also interact with macrophages through the role of cytokines such as TNF-α, which is a major secreted cytokine by macrophages. It was shown that TNF-α can enhance the anti-fungal ability of polymorphonuclear neutrophils (PMN). However, evidence on this indirect role through identification of the cytokines involved is still lacking.

NK cells play an important role against fungal infection. For example, NK cells were evidenced to inhibit the growth of Cryptococcus neoformans through a procedure similar to NK cell-mediated tumor cell lysis. However, another study suggested that it was not common for NK cells to kill the fungus directly. With activation by IL-12 and IL-18, NK cell was more prone to potentiate the nitric oxide-mediated cryptococcidal activity of thioglycolate-elicited peritoneal macrophages with the involvement of INF-γ. In addition, INF-γ and TNF-α secreted from NK cells are able to enhance the anti-fungal ability of PMN.

Although the role of NK cells against T. Rubrum infection is not well studied, a persistent T. Rubrum infection in NK deficiency patients was reported. The percentage of NK cells in the peripheral blood of the patients was significantly decreased. Furthermore, the functions of these cells were impaired. These conditions suggested inefficient fungus eradication. In another study in chronic dermatophytosis patients, an increased percentage of NK cell in sera was evidenced.
5.3.2.4 Allergens Identified in *Trichophyton Rubrum*

Lambkin et al.\(^{92}\) reported a partially purified 235,000 Dalton extracellular proteinase from *T. Rubrum*. Its activity is strongly inhibited by a metalloproteinase inhibitor together with a chymotrypsin inhibitor. The inhibition by the serine proteinase inhibitor is weak. Later, the group purified another 34,000 Dalton cell-associated proteinase from *T. Rubrum*.\(^{133}\) The enzyme has a broad substrate specificity. It can be strongly inhibited by a serine proteinase inhibitor. N-terminal amino acid sequence of the enzyme showed 50% homology with the deduced amino acid sequence of a *Coccidioides immitis* wall-associated chymotrypsin-type serine proteinase. Another study identified a 70.729 kDa heat shock protein encoded by *T. rubrum*.\(^{134}\) However, the proteinases and heat shock protein were not further characterized for their immunological potentials.

Two allergens from *T. rubrum* have been cloned. Both of them are serine proteinases. One is Tri r 2 which is a secreted alkaline peptidase with the molecular weight of about 29 kDa. This serine proteinase has a similar structure to the bacterial subtilisins. Homology is found in allergens from *Aspergillus fumigatus* (Asp f13, Asp fl 13) and *Penicillium citrinum* (Pen c13) and *Penicillium chrysogenum/notatum* (Pen ch 13). Recombinant Tri r 2 can induce both IgE-mediated reaction and cell-immunity.\(^{30}\) The other allergen, Tri r 4, is a 83 kDa prolyl oligopeptidase homologue with limited sequence identity. This peptidase bears structure similarity with the enzyme of *Aspergillus spp.*. The functions of the two allergens are not well characterized and
there are insufficient homology data.¹

5.3  Aim of Study

In the study of chapter 4 of the immunodot blot system, *Trichophyton rubrum* was shown to be the most important allergen in patients with nasal polyps/chronic sinusitis. However, the studies employing the ImmunoCAP and ELISA did not support this finding. The aim of our study is to investigate the presence of specific IgE to *T. rubrum* in nasal polyp and chronic sinusitis patients, and to characterize the possible antigenic proteins of *T. rubrum*.

5.4  Methodology

5.4.1  IgE Western Blot

5.4.1.1  Study Patients

Sera from the following groups were collected for western blot. Patient information is summarized in Table 56. Working definitions were defined in chapter 2.3.1.

I.  54 patients, 41 males and 13 females, aged from 12 to 78 years (mean age 46) with unilateral/bilateral nasal polyps, who were scheduled for functional endoscopic sinus surgery. The diagnosis of nasal polyps is based on medical history and clinical examinations, including nasal endoscopic examination and CT scan. Seven of the patients were diagnosed with asthma by respiratory physicians. All except two of the patients had concomitant chronic sinusitis.
II. 13 patients, six males and seven females, aged from 20 to 67 years (mean age 47) with unilateral/bilateral chronic sinusitis, who were scheduled for functional endoscopic sinus surgery in our department. The diagnosis of chronic sinusitis is based on medical history and clinical examinations, including nasal endoscopic examination and CT scan.

III. 17 patients, 12 males and 5 females, aged from 20 to 61 years (mean age 31) with allergic rhinitis who were scheduled for septal surgery in our department. These patients had no history of chronic sinusitis or nasal polyps. They were allergic to at least one common allergen (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, Bermuda grass, cockroach and *Aspergillus fumigatus*) tested in RAST.

IV. A control group of 48 non-rhinitis, non-atopic patients, with septal deviation who were scheduled for septal plastic surgery. Patients with nasal polyps, sinusitis, allergic rhinitis and atopy were excluded.

**Table 56.** Patient groups in the study of *Trichophyton rubrum*.

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Mean age</th>
<th>Number of patients</th>
<th>Male/Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nasal polyps</td>
<td>46</td>
<td>54</td>
<td>41/13</td>
</tr>
<tr>
<td>Chronic sinusitis</td>
<td>47</td>
<td>13</td>
<td>6/7</td>
</tr>
<tr>
<td>Allergic rhinitis</td>
<td>31</td>
<td>17</td>
<td>12/5</td>
</tr>
<tr>
<td>Control patients</td>
<td>-*</td>
<td>48</td>
<td>-</td>
</tr>
</tbody>
</table>

*, no clear information.

5.4.1.2 Reagents Used

I. Chemicals

- Tris base: Tris (hydroxymethyl)-aminomethanein, Merck KGaA
II. Solutions and gel

4x Tris.Cl/SDS, pH 6.8 (0.5 M Tris.Cl containing 0.4% SDS)
Dissolve 6.05 g Tris base (Tris (hydroxymethyl)-aminomethane, Merck KGaA)
Add in 40 ml H₂O
Adjust pH 6.8 with 1 N HCl
Add H₂O to 100 ml and filter through 0.45 µm filter
Add 0.4 g SDS

4x Tris.Cl/SDS, pH 8.8 (1.5 M Tris.Cl containing 0.4% SDS)
Dissolve 91 g Tris base in 300 ml H₂O, adjust pH 8.8 with 1 N HCl, add H₂O to 500 ml total volume and filter through 0.45 µm filter. Add 2 g SDS.

Loading dye 6 x
7 ml 4× Tris.Cl/SDS pH 6.8
1.0 (v/v) glycerol (Sigma-Aldrich Co.)
1 g SDS
0.93 g DTT (Dithiothreitol, Bio-Rad Laboratories)
1.2 mg bromphenol blue (Bio-Rad Laboratories)
Add H₂O to 10 ml
**30% acrylamide solution/0.8% bis acrylamide**

30 g acrylamide (Sigma-Aldrich Co.)  
0.8 g N, N’-methylenebisacrylamide (Sigma-Aldrich Co.)  
Add H₂O to final volume of 100 ml

**Stacking gel (3.9% acrylamide)**

0.65 ml 30% acrylamide/0.8% bisacrylamide  
1.25 ml 4× Tris.Cl/SDS, pH 6.8  
3.05 ml H₂O  
Degas under vaccum 10 to 15 min  
Add 25 µl 10%(w/v) ammonium persulfate (Sigma-Aldrich Co.)  
Add 5 µl TEMED (N,N,N’,N’-Tetramethylethylenediamine, Sigma-Aldrich Co.)

**15% separating gel**

7.5 ml 30% acrylamide/0.8% bisacrylamide  
3.75 ml 4× Tris.Cl/SDS, pH 8.8  
3.75 ml H₂O  
0.05 ml 10% (w/v) ammonium persulfate  
0.01 ml TEMED

**10x SDS electrophoresis buffer**

30.2 g Tris  
144 g glycine  
10 g SDS (Sigma-Aldrich Co.)  
Add H₂O to final volume of 1000 ml

**Coomassie blue staining solution**

50% (v/v) methanol (Fisher Scientific)  
0.05% (v/v) Coomassie brilliant blue R-250 (Sigma Chemical Co.)  
10% (v/v) acetic acid (Merck KGaA)  
40% Milli-Q H₂O

**Destaining solution**

5% methanol  
7% acetic acid  
88% Milli-Q H₂O

**Transfer buffer**

3.3 g Tris  
14.4 g glycine  
150 ml methanol  
Add H₂O to final volume of 1000 ml
**Alkaline phosphatase buffer**
100 mM Tris HCl pH 9.0
150 mM NaCl
1 mM MgCl₂

### 5.4.1.3 Protein Extraction

*T. rubrum* raw material was purchased from Allergon AB. *T. rubrum* raw material from Greer Laboratories Inc. and *Trichophyton mentagrophytes* raw material from Allergon AB were used as controls. Raw material was homogenized to suitable concentration in mortar with Trizol reagent (Invitrogen Life Technologies). Extracted solution was transferred to centrifuge tubes, 1 ml in each tube. 30 µl chloroform (Merck KGaA) was added to each tube and mixed well. After keeping the mixture at room temperature for five minutes, the mixture was centrifuged at 14000 g at 4°C for 15 minutes. Three layers formed after centrifuging. The supernatant above the middle layer was removed. 20 µl 100% ethanol (Merck KGaA) was added to every tube. After shaking well, it was centrifuged in 2000 g for 5 minutes at room temperature. The supernatant was kept and transferred to another centrifuge tube. 1.5 ml isopropanol (Sigma-Aldrich Co.) was added to every tube and kept overnight at -20°C after mixing well. The next morning, the mixture was centrifuged at 14000 g at 4°C for 15 minutes. The supernatant was removed. 2 ml 100% ethanol was added in every tube, while shaking well. The tubes were centrifuged at 14000 g at 4°C for 5 minutes. The supernatant was discarded. The tubes were then covered with parafilm with small holes. The pellet was lyophilized overnight. The lyophilized powder was suspended with 300 µl 1× loading dye and crushed. The solution was incubated at 60°C for one hour, then for 5 minutes at 100°C. The solution could be kept at -80°C until further use.
The solutions had to be centrifuged at 14000 g at 4°C for 5 minutes prior to use.

5.4.1.4 One-Dimensional SDS Gel Electrophoresis

Commercial equipment from Bio-Rad Laboratories was used for one-dimensional SDS gel electrophoresis. 15% separating gel was used. 20 µl of protein solution was loaded to every lane with 10 µl protein-marker (Bio-Rad Laboratories) in one lane. The buffer tank was then filled with 1× SDS electrophoresis buffer, followed by connecting to the power supply and running at a constant voltage of 80 V. After bromphenol blue tracking dye entering into stacking gel, the current voltage was increased to 130 V. The power supply was disconnected when bromphenol blue tracking dye reached the bottom of the separating gel. The gel was then removed from the plate and stained with Coomassie blue staining solution for one hour with slow agitation. Then the staining solution was poured out. The gel was rinsed well in distilled water and covered with destaining solution for two hours with slow agitation, until the protein bands appeared against a clear background.

5.4.1.5 Protein Transfer from Gel to Membrane

A protein transfer—Bio-Rad Criterion Blotter was used to transfer the protein from the gel to the PVDF membrane (Millipore Co.). The membrane was soaked in methanol prior to use and washed with Milli-Q H₂O. The transfer sandwich with filter paper, gel and fiber pad were prepared following instructions from Bio-Rad. The gel holder cassette was locked and put into the groove of the Criterion Blotter tank. The protein transfer was carried out at a constant voltage of 14 V overnight in a cold room.
5.4.1.6 IgE Western Blot

After transferring the protein, the PVDF membrane was removed and incubated in 5% (w/v) skimed milk powder with continuous shaking at room temperature. The membrane was then rinsed with PBS-T 0.1% (Phosphate-buffered saline with 0.1% Tween 20 from Cayman Chemical Co. Ltd., pH 7.4) three times, for ten minutes each time. Serum for test was diluted 1:1 with PBS. The membrane was incubated with serum overnight at 4°C. The next morning the membrane was washed with PBS-T 0.1% three times, ten minutes each time. The membrane was then incubated with anti-human IgE (ε chain, Sigma-Aldrich Co.) in a dilution of 1:400 at room temperature for two hours. PBS-T 0.1% was used to wash the membrane three times after incubation, ten minutes each time. NBT/BCIP color development substrate (Promega) was prepared in alkaline phosphatase buffer (pH 9.5). The membrane was immersed into NBT/BCIP for color development with about 10 to 20 minutes. After color development, the membrane was washed with distilled H2O and dried.

5.4.2 Protein Identification by Micromass Q-ToF Tandem Mass Spectrometer (Q-TOF™-MS/MS)

5.4.2.1 In-Gel Digestion

I. Reagents and Solutions

i. Reagents

- Trypsin, modified, Sequencing Grade (Promega)
- DTT (Sigma-Aldrich)
- Acetonitrile, HPLC Grade
• Iodoacetamide (Sigma-Aldrich)
• Formic acid
• Nitrogen gas, prepurified
• Water, purified to 18 MegaOhms and with removal of organics (Organex cartridge) on a Milli-Q (Millipore)

ii. Solutions

• Acetonitrile
• 100 mM ammonium bicarbonate solution
• 10 mM DTT in 100 mM ammonium bicarbonate solution (freshly made, stored under nitrogen)
• 55 mM iodoacetamide in 100 mM ammonium bicarbonate solution (freshly made, stored in darkness and under nitrogen)
• 12.5 ng/µl trypsin in 50 mM ammonium bicarbonate solution-(the Digestion solution)

II. Protocols

Finely-cut gel piece(s) was transferred to a 200 µl PCR tube. A small clean spatula was used to grind up the gel pieces coarsely. 100 µl of 50 mM NH₄HCO₃/50% (v/v) acetonitrile was added into the tube. After mixing well, the solution was removed. The above step was repeated three times followed by incubation in 50 µl acetonitrile for 5 minutes. The solvent was carefully removed with a fine gel-loading pipet tip and dried in a vacuum centrifuge. A fresh solution of 10 mM DTT in 100 mM ammonium bicarbonate was used to cover the gel fully. O₂ was removed by a flush with N₂ gas. The tube was then capped and incubated at 57°C for 60 minutes. After cooling to room temperature, the excess DTT solution was removed and 55 mM iodoacetamide
solution in 100 mM ammonium bicarbonate was added. The tube was capped after flushing carefully with N2 gas to remove O2. The tube was then wrapped in aluminum foil and kept at room temperature for 60 minutes with occasional vortexing. The solution was carefully removed using a gel-loading pipet tip. The gel was treated with 100 µl of 100 mM ammonium bicarbonate solution and mixed well. The mixture was put at room temperature for 5 minutes followed by removing the washings with pipet. The above procedure was repeated for three times. After washing, the gel was treated with 100 µl acetonitrile and mixed well. After 5 minutes, the supernatant was removed with a pipet. The above procedure was repeated twice. After that, 100 µl of 100 mM ammonium bicarbonate was added into the tube and mixed well. After 5 minutes, the supernatant was carefully removed with a pipet. 100 µl acetonitrile was added and mixed. After mixing, the solvent was carefully removed. The above procedure was repeated twice. The gel was then dried in a vacuum centrifuge, followed by incubation with 15-30 µl of digestion solution (12.5 ng/µl trypsin in 50 mM ammonium bicarbonate solution) was at 40°C for 30 minutes. The excess trypsin solution was carefully removed, followed by incubation in 15 µl of 50 mM ammonium bicarbonate solution overnight at 37°C. The next morning, the gel was cooled to room temperature and centrifuged at 6000 g for 10 minutes. The supernatant was carefully moved with a pipet and saved. The gel was treated with 20 mM ammonium bicarbonate and mixed well. The mixture was centrifuged at 6000 g. After centrifuging, the supernatant was moved with a pipet and saved. The gel was then treated with 5% formic acid in 50% aqueous acetonitrile (10-25 µl). The mixture was
allowed to stand for 5 to 10 minutes and centrifuged at 6000 g. After that, the supernatant was moved with a pipet and saved. The above procedure was repeated twice. The three supernatants obtained was combined and dried in a vacuum centrifuge.

5.4.2.2 Purification and Concentration of Peptides Isolated from In-Gel Digestion of Proteins by Zip-Tip

I. Preparation of Zip-Tip C18 Cartridge Column

There were altogether 7 tubes (A to G) used in this procedure. They were:

- Tube A: Wetting Solution (100 µl acetonitrile, HPLC Grade).
- Tube B: Cleaning Solution (1000 µl water, HPLC Grade).
- Tube C: 10-20 µl sample solution. The solution was prepared by dissolving the extracted peptides in 10-20 µl 0.5% formic acid. The lower sides of the PCR tube containing the extracted peptides had to be carefully washed to achieve maximum dissolution of the peptides.
- Tube D: Washing solution (500 µl 0.5% formic acid)
- Tube E: First extraction solution (100 µl 0.5% formic acid in 1:1 (v/v) water : acetonitrile)
- Tube F: Second extraction solution (100 µl acetonitrile)
- Tube G: A clean empty 200 µl PCR tube.
II. **Protocols**

The Zip-Tip column was attached to the 20 μl micropipet (e.g. Gilson's Pipetman P20) snugly. The volume setting was adjusted to 7 μl. Acetonitrile (Tube A) was carefully withdrawn through the Zip-Tip. The acetonitrile was carefully removed with pipet while the tip of the Zip-Tip was still under the acetonitrile. This step was repeated at least 7 to 8 times, or until no bubbles arised during the pipetting out step. Finally, the acetonitrile was pipetted out slowly. While the plunger was still down, the tip of the Zip-Tip was immersed into the water (Tube B). 7 μl of water was withdrawn through the Zip-Tip slowly and pipetted out carefully. This step was repeated at least 10 times to ensure washing away all acetonitrile. The water was then pipetted out. While the plunger was still down, the tip of the Zip-Tip was moved into the sample solution (Tube C). After carefully filled with the sample solution, the Zip-Tip was pushed out into Tube C carefully. The above procedure was repeated at least 10 times to ensure that most of the peptides were retained on the Zip-Tip. The Zip-Tip was washed with 0.5% formic acid solution (Tube D) in the same fashion in the above steps to desalt and wash the peptides. The wash solution was then pipetted out. When the plunger was still down, the Zip-Tip was transferred to Tube E (extraction solution) and waited for 20 seconds. The extracting solution (Extract 1) was then pipetted out into Tube G. While the plunger was still down, the extracting solution was moved to the Tube F containing the acetonitrile. The acetonitrile was then slowly withdrew through the Zip-Tip. After waiting for 10 seconds, the solution (Extract 2) was pipetted out into
Tube G containing the Extract 1. The combined extract (Tube G) was then submitted for Q-TOF™-MS/MS analysis.

5.4.3 N-terminal Sequencing of Purified Proteins by High-Performance Liquid Chromatography (HPLC)

5.4.3.1 Protein Recovery from Polyacrylamide Gels

The gel slice containing the band of interest was minced as finely as possible in a microcentrifuge tube. One hundred ml of gel elution buffer (50 mM ammonium bicarbonate from Sigma-Aldrich Co., 0.1% SDS, pH 7.8) was added. The gel slice was incubated at 37°C for at least one hour. After centrifugation for 2 minutes in a microcentrifuge, the supernatant was transferred to another microcentrifuge tube and the acrylamide fragments were washed with 100 ml of gel elution buffer. The two supernatants were pooled. The dye and residual SDS bound to the protein precipitate were removed by adding 100 ml of cold (-70°C) 80% acetone. The remaining solution was placed at -20°C for 5 minutes and centrifuged in a microcentrifuge for 2 minutes at 4°C. The pellet was lyophilized and resuspended in 100 ml MilliQ water.

5.4.3.2 Reduction and Alkylation of Proteins

The protein was dissolved in one ml of 6 M guanidine-HCl (Sigma-Aldrich Co.) with 0.25 M Tris-HCl and 1 mM EDTA (pH 8.5, Sigma-Aldrich Co.). Two ml of 2-mercaptoethanol (Sigma-Aldrich Co.) was added in followed by incubation at 37°C for two hours under nitrogen. Two ml of 4-vinylpyridine (Sigma-Aldrich Co.) was added, mixed, and incubated at 37°C for another two hours under nitrogen.
5.4.3.3 Desalinization of Protein by Size Exclusion Chromatography

The size exclusion chromatography column was packed with Bio-Gel P-4 gel [medium, 130 ±40 µm, and nominal exclusion limit of 800-4,000 dalton (BIO-RAD Laboratories, UK)]. The elution buffer used was 90% formic acid.

5.4.3.4 Separation of Reduced and Alkylated Proteins by Reverse-Phase HPLC and N-terminal Sequencing

An Amersham Pharmacia Biotech reversed-phase high-performance liquid chromatograph was used to determine the profiles of the reduced and alkylated proteins recovered from SDS-gel. The column is Hi™Prep 16/60 Sephacryl™ S-200 High resolution prepacked gel filtration (size exclusion) column. The elution buffers were buffer A 0.1% [trifluoroacetic acid (Ferak, Berlin, F.R.G.)], and buffer B [80% acetonitrile (Rathburn Chemicals, Walkerburn, U.K.)], with doubly glass-distilled water. The UV detection was under 260 nm, 280 nm and 215 nm with a flow rate of 0.2 ml/min. The purified proteins were sent for N-terminal sequencing by the ABI Procise® 494 protein sequencer. The sequencing result was combined with the result of Q-TOF™-MS/MS and aligned to the National Center for Biotechnology Information (NCBI, NIH) Genebank sequence using the BLAST (Basic Local Alignment Search Tool) program.

5.5 Results

5.5.1 One-Dimensional SDS Gel Electrophoresis

Figure 38 illustrates a typical protein separation pattern of T. rubrum raw material from two different batches of Allergon AB (A, B) and from paper disc of RidaScreen.
ELISA kit for *T. rubrum* (C). Different protein profiles can be seen in raw materials even from the same company. In the one-D electrophoresis of proteins extracted from paper disc of RidaScreen ELISA kit for *T. rubrum*, bands were too weak to be seen, suggesting an unreliable test result.

![Coomassie blue staining of one-D electrophoresis](image)

Figure 38. Coomassie blue staining of one-D electrophoresis of extraction of *T. rubrum* from two batches purchased from Allergon (A, B) and protein extracted from paper disc of ELISA kit used in chapter 4 (RidaScreen).

5.5.2 IgE Western Blot of the Study Groups

Table 57 shows the percentages of the reactive sera to *T. rubrum* extraction in different study groups, with the bar chart shown in Figure 39. Over 90% of the sera from nasal polyp and chronic sinusitis patients were reactive to the protein extraction of *T. rubrum*. That percentage was relatively higher than the percentage of 76% in allergic rhinitis patients, and remarkably higher than that of the controls (19%). The 15 kDa band was the major reactive band followed by the 60 kDa one in all the
groups. They may have existed together or individually. The nasal polyp group had the highest percentage of the total reactive sera. This group also had the highest percentage of 15 kDa reactive, 60 kDa reactive, and both 60 and 15 kDa reactive sera compared to other groups. Four patients in the nasal polyp groups also reacted to the 10 kDa band together with the 15 kDa band. Sera from chronic sinusitis patients were mainly reactive to the 15 kDa band. Compared with the other three patient groups, the reaction of the control group was relatively weak. **Figure 40 (I to IV)** shows the results of IgE western blot in our study groups.

![Figure 39](image_url)

**Figure 39.** Bar chart of the percentage of reactive sera to the 15 kDa and/or 60 kDa proteins from extraction of *T. rubrum* in different study groups. NP, nasal polyp patients; SI, chronic sinusitis patients; AR, allergic rhinitis patients; CON, controls.
### Table 57. Percentage of sera reactive to *T. rubrum* extraction (15 kDa and 60 kDa) in nasal polyp patients (n=54), chronic sinusitis patients (n=13), allergic rhinitis patients (n=17) and controls (n=48).

<table>
<thead>
<tr>
<th></th>
<th>Reactive Sera</th>
<th>15 kDa positive</th>
<th>60 kDa positive</th>
<th>15 and 60 kDa positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nasal Polyps (n=54)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>51 (94%)</td>
<td>46 (85%)</td>
<td>35 (65%)</td>
<td>30 (56%)</td>
</tr>
<tr>
<td>Chronic Sinusitis (n=13)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12 (92%)</td>
<td>12 (92%)</td>
<td>2 (15%)</td>
<td>2 (15%)</td>
</tr>
<tr>
<td>Allergic Rhinitis (n=17)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>13 (76%)</td>
<td>11 (65%)</td>
<td>6 (35%)</td>
<td>4 (24%)</td>
</tr>
<tr>
<td>Controls (n=48)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>9 (19%)</td>
<td>9 (19%)</td>
<td>3 (6%)</td>
<td>3 (6%)</td>
</tr>
</tbody>
</table>

**Figure 40 (I to IV).** IgE western blot to *Trichophyton rubrum* of nasal polyp patients (n=54), chronic sinusitis patients (n=13), allergic rhinitis patients (n=17) and controls (n=48).

I. Nasal polyp patients (n=54)
Figure 40, Continued.

I. Nasal polyp patients (n=54), continued,
Figure 40, Continued.

II. Chronic sinusitis patients (n=13)

III. Allergic rhinitis patients (n=17)
Figure 40, Continued.

III. Allergic rhinitis patients (n=17), continued.

IV. Controls (n=48)
Figure 40, Continued.

IV. Controls (n=48), continued.
5.5.3 IgE Western Blot to *Trichophyton rubrum* from Different Companies

**Figure 41 (A,B).** Western blot of selected sera to extraction of *T. rubrum* and *T. mentagrophytes* from Allergon AB and Greer Laboratories. TR(A)₁, *T. rubrum* (Allergon). TR(G)₂, *T. rubrum* (Greer). TM(A)₃, *T. mentagrophytes* (Greer).

Figure 41 (A, B) shows the results of IgE western blot to extraction of *T. rubrum* from different companies of selected sera. Reactions to raw material of *T. mentagrophytes* were compared in some tests. The reactions of sera to extracted proteins of *T. rubrum* from different companies and different batches were always consistent although the protein files were not exactly the same. Controls of *T. mentagrophytes* showed much weaker reactions compared with *T. rubrum*. In addition, the reactions between the two species showed different protein patterns, suggesting no cross-reactions between them.

5.5.4 Q-TOF™-MS/MS, HPLC and N-terminal Sequencing

The 15 kDa and 60 kDa bands were first sent for N-terminal sequencing. Because the protein files are complicated, their identification is far from obvious. So we use Q-TOF™-MS/MS to repeat the analysis the protein bands. However, the database search
failed to detect any homology. Proteins recovered from the two bands were further purified by HPLC and sent again for N-terminal sequencing. Unfortunately, amino acids sequence determined by N-terminal sequencing was quite short. N-terminal sequencing of the other protein peaks failed. Figures 42 and 43 show the HPLC purification profiles of the proteins recovered from the 15 kDa and the 60 kDa protein bands, respectively. Proteins with amino acids sequence determined by N-terminal sequencing are indicated. These results were combined with the result from Q-TOF™-MS/MS for BLAST. Table 58 shows the main result of N-terminal sequencing and Q-TOF™-MS/MS of the proteins recovered from the 15 kDa and 60 kDa bands of *T. rubrum* extraction. They were taken together for sequence alignment in NCBI by BLAST.

**Table 58.** Main results of N-terminal sequencing and Q-TOF™-MS/MS of the proteins recovered from the 15 kDa and 60 kDa bands of *T. rubrum* extraction.

<table>
<thead>
<tr>
<th>N-terminal sequencing</th>
<th>Q-TOF™-MS/MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 kDa</td>
<td></td>
</tr>
<tr>
<td>SGE</td>
<td>946.3522</td>
</tr>
<tr>
<td>DSGAHY</td>
<td>1653.7122</td>
</tr>
<tr>
<td>SGA</td>
<td></td>
</tr>
<tr>
<td>STY</td>
<td></td>
</tr>
<tr>
<td>60 kDa</td>
<td></td>
</tr>
<tr>
<td>DTY</td>
<td>1408.6821</td>
</tr>
<tr>
<td></td>
<td>1445.6222</td>
</tr>
<tr>
<td></td>
<td>1159.5222</td>
</tr>
<tr>
<td></td>
<td>1456.7222</td>
</tr>
</tbody>
</table>
Figure 42. HPLC purification profiles of proteins recovered from the 15 kDa protein band of *T. rubrum*. Peaks with amino acids sequence determined by N-terminal sequencing are indicated.
Figure 43. HPLC purification profiles of proteins recovered from the 60 kDa protein band of *T. rubrum*. The peak with amino acids sequence determined by N-terminal sequencing is indicated.
5.5.5 Homology Identified by BLAST

The following homologous proteins were identified,

I. Protein Recovered from 15 kDa Gel Slice

Proteins in the 15 kDa band was homologous with the 1, 3-beta-glucanosyltransferase from *Gibberella zeae* (Figure 43) with identities of 50%.

*T. rubrum* 15 kDa: 10  DSGAHYLLDMTNPK 23  
*Gibberella zeae* glucanosyltransferase:  591 DAGIYLVLDVNNPK 632  

**Figure 44.** Alignment of amino acid sequence of proteins recovered from the 15 kDa gel slice of *T. rubrum* and 1, 3-beta-glucanosyltransferase from *Gibberella zeae*.

1, 3-beta-glucanosyltransferase of *Gibberella zeae* has the function correlated with biogenesis of cell wall (cell envelope). Its theoretical pI is 8.0 with molecular weight of 23 kDa. If the signal peptide is removed, the pI and molecular weight will be 7.55 and 22 kDa, respectively. It may be glycosylated. **Figure 45** shows the signal peptide as well as the O-glycosylated sites of 1, 3-beta-glucanosyltransferase of *Gibberella zeae*.

MKSVSLLSVLAVASA  
TPTLKEPPKRGSLPTVASSNAdvAEGDERFYLQGIDYQPGGASANEDPLADPKVCK  
RDIKYKELGVPVRVYAVDNKADHDECMLDDAGIYLVLDVNNPKSYINRANNGP  
SYNAAYIQSVPATVEMFAQYENTLAFSSGNEVMNDEKTDKSAPYVKAITRDNRNYI  
KARKLRKIPVXYSAADVASTACRRP  

**Figure 45.** Signal peptide (highlighted letters with yellow color) and O-glycosylated sites (highlighted letters with green color) of 1, 3-beta-glucanosyltransferase of *Gibberella zeae*.

II. Proteins Recovered from the 60 kDa Gel Slice

Two homologous proteins were identified. They are stress-inducible protein (sti35) of *Gibberella zeae* and *Fusarium oxysporum*.
i. **Homology with the stress-inducible protein (sti35) of Gibberella zeae**

The homologous protein identified in *Gibberella zeae* (Figure 46) is stress-inducible protein (sti35) with identities of 51%. It is a thiazole biosynthetic enzyme which is a mitochondrial precursor. The theoretical pI of sti35 in *Gibberella zeae* is 6.01 with molecular weight of 35 kDa. It does not have a signal peptide. It may be glycosylated.

**Figure 47** shows the O-glycosylated sites and homology identified with *T. rubrum*.

<table>
<thead>
<tr>
<th>T. rubrum 60 kDa:</th>
<th>G. zeae sti35:</th>
</tr>
</thead>
<tbody>
<tr>
<td>SGLKAAEDALLG_</td>
<td>SGLKAEAL_</td>
</tr>
<tr>
<td>GEGVGGHDTRK_</td>
<td>KI_</td>
</tr>
<tr>
<td>27</td>
<td>315</td>
</tr>
</tbody>
</table>

**Figure 46.** Alignment of amino acid sequence of proteins recovered from the 60 kDa gel slice of *T. rubrum* and stress-inducible protein (sti35) from *Gibberella zeae*.

Figure 47. O-glycosylated sites (highlighted letters with yellow color) in the amino acid sequence of sti35 of *Gibberella zeae* (322aa). Green highlighted letters are homologous with *T. rubrum*.

ii. **Homologous proteins with stress-inducible protein (sti35) of Fusarium oxysporum**

Another homologous protein identified is the stress-inducible protein (sti35) of *Fusarium oxysporum* (Figure 48 and Figure 49) with identities of 51%. It may be glycosylated. **Figure 49** shows the O-glycosylated sites and homology identified with *T. rubrum*. 

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Figure 48. Alignment of amino acid sequence of proteins recovered from the 60 kDa gel slice of T. rubrum and stress-inducible protein (sti35) from Fusarium oxysporum.

Figure 49. Amino acid sequence of sti35 of Fusarium oxysporum (320 aa). Homologous with T. rubrum is indicated (green highlighted letters). Yellow highlighted letters are O-glycosylated sites.

5.6 Discussion

Fungi are important outdoor and indoor allergens. The most important species related with nasal polyps and chronic sinusitis are Aspergillus fumigatus, Candida spp., Alternaria alternata, Bipolaris spp. and Curvularia lunata.6-9 The role of fungi in the pathogenesis of nasal polyps and chronic sinusitis remains controversial. The incidence of fungi identified in nasal and sinus cavities from chronic sinusitis and/or nasal polyp patients ranges from 7% to 93%.5,10-12 Although fungal pathogens are frequently identified in specimens from chronic sinusitis and/or nasal polyp patients, they are also commonly found in normal controls, suggesting the variable effect of fungi in different individuals.5,10 Whether or not fungi contribute to the pathogenesis of nasal polyps and chronic sinusitis through the role of IgE-mediated allergy is under debate. Although eosinophilia is predominant in the patients, the evidence of IgE-mediated allergy is lacking.5,10
5.6.1 Evaluation of ImmunoCAP and Commercial ELISA Kit by Western Blot

In the study of immunoarray dot blot system discussed in chapter 4, we identified a remarkably high incidence rate, i.e., 90% and 80%, of serum specific IgE to *T. rubrum* in nasal polyp and chronic sinusitis patients, respectively. However, there was no concordance with the results of the ImmunoCAP system and ELISA kit test. Western blot of sera from nasal polyp and chronic sinusitis patients to *T. rubrum* protein extraction proved that over 90% of the sera were positive. Consistent reactions to *T. rubrum* were found even though protein electrophoresis from different companies or batches from the same company were not the same. Proteins in the 15 kDa and 60 kDa bands are the ones with the strongest reaction and highest incidence in all the study groups. In addition, there was no cross-reactivity between *T. rubrum* and *T. mentagrophytes* in our study. The agreement between immunoarray blot system and western blot, as compared to the disagreement between immunoarray blot system and ImmunoCAP or ELISA, suggests that commercial tests for fungal allergens are not yet optimal and need far more further standardization.

5.6.2 Role of *T. Rubrum* in the Pathogenesis of Nasal Polyp and Chronic Sinusitis

5.6.2.1 Nasal Polyps and Chronic Sinusitis, Does *T. Rubrum* Exert Its Role?

*T. rubrum* is the most common dermatophyte related with skin and nail infections worldwide. It often causes a superficial infection, quite different from deep infections caused by *Aspergillus spp.* and *Candida spp.* It is often found in patients with
refractory and recurrent infection or with immunosuppressed diseases.

Davies et al.\textsuperscript{135} reported that nasal symptoms (sneezing, itching and blocking) were found in 72\% of the patients who complained of being troubled by nasal dust, in which \textit{T. rubrum} was the commonest dermatophyte. Although \textit{T. rubrum} has been reported in patients with trychophytosis, asthma and rhinosinusitis,\textsuperscript{82,86,87} studies in nasal polyps and chronic sinusitis reported that the incidence rate of positive culture of \textit{T. rubrum} was only 1\% to 2\%.\textsuperscript{5,8} In a study Karlsson et al.\textsuperscript{136} reported that the common features of a group of patients with dermatophytosis, asthma and/or perennial rhinitis were: positive RAST to \textit{Trichophyton tonsurans}, positive skin test to mixture of three \textit{Trichophyton} species; increased severity of symptoms to nasal challenge with increased \textit{Trichophyton} extract; increased level of tryptase and histamine in nasal lavage after nasal challenge; negative fungal culture from nasal swabs; and absence of fungal hyphae in nasal lavage. However, 50\% of the patients had chronic sinusitis, as defined by radiographic examination and half of them had earlier operations done on the sinusitis. Therefore, it seems that \textit{Trichophyton} may enter a sinus or the nasal cavity, not through direct airway exposure but through systemic absorption from the skin. Other studies proposed that \textit{T. rubrum} entered the airway through systemic absorbance or inhalation of dust from nail clipping.\textsuperscript{82,86,87}

In a recent study Gutman et al.\textsuperscript{6} identified the incidence rate of positive skin prick test to \textit{Trichophyton spp.} at 23.1±11.7\% in chronic sinusitis patients. A positive test was
only found in those with seasonal but not perennial allergy. This incidence, although
higher than in other reports, is still not comparable to that of *Aspergillus* spp.,
*Candida* spp. and *Alternaria* spp.. Singapore has a typical tropical climate. The
temperature is constant all year around between 24°C to 32°C. The daily average
relative humidity is as high as 84.4%. This unique climate is perfect for fungus, which
grows best between 18°C to 32°C and with essential relative humidity above 65%.
Even though specific IgE to fungus is commonly identified in nasal polyp, chronic
sinusitis and allergic rhinitis patients, the fungus species involved were not the same.
As we identified in the immunoarray dot blot system, in nasal polyp and chronic
sinusitis patients, *T. rubrum* gave the highest incidence. In allergic rhinitis patients,
*Penicillium expansum* was the most common species. Thus, both environmental
factors and host factors affect the outcome of a fungus infection greatly.

5.6.2.2 Immunodeficiency and Atopy, Predisposing Factors for Chronic Infection
of *T. Rubrum* in Nasal Polyps and Chronic Sinusitis?

The host’s natural immune system is able to reduce or eliminate the pathogen and
limit the spread of fungus infections, especially superficial infections such as those
caused by *T. rubrum*. In patients with immunosuppressive diseases, a fungal infection
may become chronic and widespread.

Although immunodeficiency is accepted as a risk factor in the development of nasal
polyps and chronic sinusitis, there are very few reports on the immunosystem function
in nasal polyp and chronic sinusitis patients. Chee et al. evaluated the immune dysfunction in patients with refractory sinusitis. Patients with HIV, AFS, CF, and primary ciliary dyskinesia were excluded. The study group identified a surprisingly high incidence rate of immune dysfunction in the patients. 40% of the patients were anergic in DTH test. 54.8% showed abnormal proliferation in response to recall allergens. 11.3% had a decreased response to alloantigen and 26.3% had a decreased response to T cell mitogens. Low IgG, IgA and IgM were identified in 17.9%, 16.7% and 5.1% of the patients, respectively. Common variable immunodeficiency and selective IgA immunodeficiency were identified in 9.9% and 6.2% of the patients, respectively. Although immuno dysfunction is clearly a predisposing factor of fungus infections, whether it is a common phenomenon in chronic sinusitis with/without nasal polyps need to be further investigated.

Besides immunodeficiency, atopy has also been suggested to predispose for persistent infection of *T. rubrum*, especially in those patients with atopic respiratory disease. However, atopy is not a universal phenomenon in nasal polyps and chronic sinusitis and has no correlation with local inflammation as we discussed in chapter 2. Interestingly, in our study patients with allergic rhinitis who had a well defined atopy status, the percentage of patients with specific IgE to *T. rubrum* was lower than in nasal polyp and chronic sinusitis patients. It seems that in deciding the predisposition of a *T. rubrum* infection, there may exist more complicated host factors than atopy alone.
5.6.2.3 Suppressed Cell Immunity and Chronic *T. rubrum* Infection: What is the Relationship?

Compared to those patients with non-chronic infection of *T. rubrum* who mainly show positive DTH, patients with a chronic infection are skin anergic or mainly show immediate hypersensitivity. Lack of DTH suggests suppressed cell-mediated immunity. However, most of the studies have evidenced normal cell immunity in patients with chronic trychophytosis. Therefore, suppressive factors such as suppressor T cells and macrophages which alter the response of cell-mediated immunity, or defects of the cell-mediated immunity may contribute to this phenomenon.

Circulating antigens were identified in patients with *T. rubrum* infections, especially in those with chronic infection or defective cell-mediated immunity. These antigens are supposed to modulate the cell-mediated immune response of the hosts. Mannan component of the *T. rubrum* cell wall (TRM) can bind with monocytes, and thus inhibit cell-immunity through inhibition of antigen presentation and antigen processing. In addition, xanthomegnin was identified in epidermal materials infected with *T. rubrum*. Xanthomegnin is derived from the polyketide chemical pathway which gives rise to aflatoxin, a mycotoxin produced by *Aspergillus spp.*. Aflatoxin was proved to impair phagocytosis by alveolar macrophages. In addition, it may cause defects in humoral immunity, cell-mediated immunity and natural killer cell dysfunction through the inhibition of IL-2, INF-γ, TNF-α and IL-1α. Whether
xanthomegnin plays similar roles as aflatoxin has not been demonstrated.

As we discussed in chapters 2 and 3, nasal polyp tissue and inflamed sinus mucosa are characterized by increased CD8+ T cell levels and an inverse CD4+/CD8+ T cell ratio. Whether CD8+ T cell contributes to the lack of DTH as suppressor T cell needs to be further evaluated.

Cell immunity impairment has been suggested in nasal polyp and chronic sinusitis patients. Scheeren et al.\textsuperscript{142} reported that in patients with/without nasal polyps but having normal ciliary functions, 75% showed defective DTH to candidal antigen or streptokinase-streptodornase antigen. These patients showed decreased functional chemotaxis as well. They suggested that rhinoviral p15E-like protein may play a partial role in the decreased cell immunity in nasal polyp and chronic sinusitis patients. Suppressed cell immunity is clearly a risk factor for infection. Although impaired cell immunity has been identified in nasal polyps and chronic sinusitis, the underlying conditions have not been studied well. Whether nasal polyp and chronic sinusitis patients commonly have primary impaired cell immunity, or this impaired immunity is correlated with pathogens, such as \textit{T. rubrum}, is not known.

5.6.2.4 The Role of Innate Immunity in Nasal Polyps and Chronic Sinusitis with Chronic \textit{T. Rubrum} Infection

Innate immunity, including PMNL (polymorphonuclear leukocytes, i.e., eosinophils,
neutrophils and basophils), monocytes/macrophages, and NK cells, is considered to play an important role against fungus infection.\textsuperscript{123} Although phagocytes may kill fungal pathogens through phagocytosis, it was suggested that neutrophils and monocytes mainly kill a fungus by the generation of cytotoxic oxygen.\textsuperscript{127,128} Complement system activation and inflammatory cell aggregation, on one hand, help to eradicate pathogens; on the other hand, they initiate inflammation.

*T. rubrum* has been proven to be a potent complement system activator.\textsuperscript{124} Neutrophil chemotactic factor (NCF) production by C5a activation will recruit neutrophils.\textsuperscript{125,126} As we discussed in chapter 3, chemokines derived from neutrophils have been shown to have potential roles in chemotactic and activating NK cells.\textsuperscript{143-145} We have demonstrated the infiltration of neutrophils and NK cells in nasal polyp tissue and inflamed sinus mucosa (chapters 2 and 3). The NK cell will then exert its role in both innate immunity and adaptive immunity. Besides the well defined role in innate immunity, NK cells regulate other inflammatory cells through direct or indirect interactions. By lysis of dendritic cells or macrophages, they affect antigen presentation.\textsuperscript{146} By interaction between 2B4 (CD244) and CD48, they enhance proliferation and activation of CD4+ and CD8+ T cells as well as their own cytotoxicity and INF-γ production.\textsuperscript{147} Cytokine secretion by NK cells, not only INF-γ and TNF-α\textsuperscript{148} but also IL-5,\textsuperscript{149} will lead to a cytotoxic T cell response or an eosinophilic allergic inflammation.
5.6.2.5 Does *T. Rubrum* Act as Superantigen?

Schubert et al.\(^{19}\) proposed that chronic hypertrophic sinusitis, AFS and other related disorders, such as chronic severe asthma, are all chronic eosinophilic-lymphocytic respiratory mucosal inflammatory disorders induced by superantigen-producing extra- or intracellular microbes. Two factors are essential for the onset and persistence of this chronic inflammation: the genetic make up of the host T cell receptor V\(\beta\) and the persistent superantigen production/exposure at the respiratory mucosa. *Alternaria*, a dematiaceous fungus frequently cultured from nasal polyp and chronic sinusitis patients, stimulated peripheral blood lymphocytes from hyperplasia chronic sinusitis patients, but not normal controls in vitro.\(^{19}\) In addition, the coexistence of specific IgE to dematiaceous fungi and *S. aureus* is almost an universal phenomenon.\(^{150}\) Therefore, it was proposed that hyperplasia chronic sinusitis patients may have TCR V\(\beta\) motifs which can bind superantigens from both *S. aureus* and dematiaceous fungi.\(^{19}\)

Although TCR V\(\beta\)13 motif has been identified in ABPA,\(^{19}\) evidence for the recently raised theory of superantigen induced eosinophilic-lymphocytic respiratory mucosal inflammatory disorders is still lacking.

Kanda et al.\(^{107}\) investigated chemical mediators released by peripheral blood mononuclear cells (PBMC) in atopic dermatitis (AD) patients, psoriasis vulgaris and normal controls induced by *Malassezia furfur* (MA), *Candida albicans* (CA) and *T. rubrum*. It was interesting that *T. rubrum* only induced IL-4 release in AD patients which was different from the other two allergens. This suggests that *T. rubrum*
initiates T cell immune response through a unique way. In addition, dermatophytosis caused by *T. rubrum* often shows interplay of fungus and bacteria, such as *S. aureus*, *M. sedantarius*, *B. epidermidis*, *C. minutissimum*, *Corynebacterium jeikeium*, *Pseudomonas* and *Proteus spp.*[^109] The growth of *T. rubrum* produces a penicillin- and streptomycin-like substance which favors secondary antibiotic-resistant bacteria infections.[^151] On the other hand, bacteria will cause tissue damage by secretion of a proteolytic enzymatic substance.[^109] The interaction between dermatophyte and bacteria usually results in a low recovery rate from the related diseases. In addition, some bacteria, such as *M. sedantarius* and *B. epidermidis* are able to produce sulfur compounds which are potent antifungal agents.[^109] In this case, the dermatophyte will be suppressed, and as a consequence, the primary dermatophyte-positive interspaces will become converted into macerated interspaces. Although microorganisms, especially *Staphylococcus aureus*[^152,153] are commonly identified in nasal polyp tissue and inflamed sinus mucosa, and *Staphylococcal* enterotoxins were suggested to contribute to the development of nasal polyps as a bacterial superantigen,[^150] whether the findings in trichophytosis can also be identified in nasal polyp tissue or chronic sinusitis remains to be investigated.

The high incidence rate of specific IgE to *T. rubrum* in nasal polyp and chronic sinusitis patients was proved by western blot, although it was not supported by ImmunoCAP or ELISA kit. It is interesting to note that we did not identify infiltration of langerhans cells or B cells in nasal polyp tissue and inflamed sinus mucosa. *T.
rubrum seemed to activate T cells with little help from APCs, suggesting the possible role of superantigens. Other possibilities may also exist. Besides the mechanisms discussed in chapter 2, the production of *T. rubrum* specific IgE may be caused by primary dermatophytosis. As we discussed in chapter 3, NK cells may lyse dendritic cells and macrophages, leading to a decreased number of APCs. The mechanism to explain the discrepancy between the production of specific IgE to *T. rubrum* and the rarely seen langerhans cells or B cells in nasal polyps and chronic sinusitis needs further study.

### 5.6.2.6 Functions of Allergens from *T. Rubrum*

Homologous proteins to allergens in *T. rubrum* are identified in *Fusarium* spp., a filamentous fungus commonly known as plant pathogen as well as an important airborne allergen to humans. However, homologous information was quite limited due to the short amino acids sequence determined by N-terminal sequencing. Success of N-terminal sequence is limited by several factors. First, a successful sequencing needs sufficient amount of material. Another important factor is that proteins may be blocked naturally or during the process of purification. Most eukaryotic cells (at a rate of 70% or greater) are blocked as a result of posttranslational modification. In such cases, attemptation has to be made to deblock the protein or try internal sequencing. Large amount of material is needed for fragmentation of the protein, followed by isolation and sequence analysis of the peptides. Internal amino acid sequence analysis can help us to identify the nature and the site of posttranslational modifications;
identify the site of a group specific reagent, or affinity label; and determine the complete amino acid sequence of a protein.

The theoretical molecular weight of $1,3$-beta-glucanosyltransferase of *Gibberella zeae* is 23 kDa, while the theoretical molecular weight of sti35 from *Gibberella zeae* and *Fusarium oxysporum* is 35 kDa. These proteins all have O-glycosylated sites. There may be discrepancy between theoretical and apparent molecular weight determined by one-D electrophoresis because of O-glycosylation. The existence of high pI value and unusually high proportion of basic or charged amino acids is also associated with abnormal migration.

*Fusarium spp.* has been identified in chronic sinusitis. It is also one of the most important fungi producing mycotoxins, including fumonisin, and trichothecenes which have a high immunosuppressive function. In a recent report on exposure to toxic fungi by Rea et al., *Fusarium spp.* was found in 50 out of 69 (73%) houses studied, suggesting a common distribution in the environment. 22 out of the 24 (91%) patients exposed to molds showed a positive intradermal test to *Fusarium*. The incidence rate of positive intradermal tests to trichothecenes was even higher, at a percentage of 100%. Besides strong evidence of mycotoxin exposure, patients commonly showed symptoms in the airway, the nervous and the immune system, suggesting systematic involvement. Physical examinations showed abnormal lymphocyte subset counts in many patients. 71% patients showed suppressed cell immunity.
The homologous proteins to allergens in *T. rubrum* are stress-inducible proteins of *Fusarium graminearum* (*Gibberella zeae*) and *Fusarium oxysporum*, and glucanosyltransferase of *Fusarium graminearum*. In the study of the immunoarray system ([chapter 4](#)), we also test specific IgE to *Fusarium solani* and *Fusarium moniliforme*. The incidence of specific IgE to *Fusarium solani* was 22% in nasal polyp patients and 57% in chronic sinusitis patients. No patients were identified with specific IgE to *Fusarium moniliforme*. This result is not comparable to that of *T. rubrum*, suggesting a low chance of cross reactivity.

Heat shock proteins (Hsps) or stress proteins are highly conserved both in prokaryotes and eukaryotes. Their expression is enhanced by many factors such as change of environment, viral or bacteria infection and toxin exposure. Hsps have three basic functions: to act as molecular chaperones, preventing misaggregation of denatured proteins or helping refolding of denatured proteins; regulating cellular redox state; regulating protein turnover. Hsps are classified according to their molecular weight. The well studied groups are Hsps with molecular weight of 110, 90, 70, and 60 kDa. Other Hsps include 34, 47, 56, 75, 78, 94, 174 kDa and those with small molecular weight about 20 kDa. Hsp70 has been successfully cloned from *T. rubrum*, although its biological functions remain unknown. Sti35 is a heat shock protein with molecular weight of 35 kDa. Sti35 in *Fusarium* has been successfully cloned. Homologues to sti35 were identified in varieties of eukaryotes such as *S. cerevisiae*, *Aspergillus spp.* and soybean, suggesting the correlation between these
homologous proteins and unknown basic biological functions in eukaryotes. Ethanol is a potent inducer of sti35 but disruption of sti35 does not affect the growth of Fusarium at various temperatures. Hsps, mainly Hsp 60 and Hsp 70, have been proven to induce an effective immuno response, including activation of monocytes, epithelial and endothelial cells, activation of cytotoxic T cell through dendritic cell presentation, NK cell attraction and activation, and hypersensitivity. However, so far, the function of sti35 is not clear.

Fungal cell wall is not only essential for its survival but also efficiently initiates immuno response in the host. It has a highly dynamic structure with glucan (β-1, 3-glucan, β-1, 6-glucan, α-1, 3-glucan) as a skeleton and chitin and mannoproteins anchored onto it. 1, 3-β-glucanosyltransferase is a glycosylphosphatidylinositol (GPI) protein involved in 1, 3-β-glucan assembly to form functional cell walls of fungi. Homologues has been identified in Aspergillus famigatus, Saccharomyces cerevisiae and Candida albicans. A recombinant 1, 3-β-glucanosyltransferase has also been successfully applied in animal model against fungal infection. Glucan has been proved to induce innate immunity and cytokine production, mainly INF-γ and TNF-α. However, mannoprotein which forms the outer layer of the fungi cell wall is supposed to be the most important antigen. Although direct evidence of the antigenicity of glucan in humans is still lacking, a Th2 predominant response induced by inhalation of the β-1, 3-glucan in murine models has been shown. It was also reported that β-1, 3-glucan rather than endotoxin in house dust was closely
related with asthma severity.\textsuperscript{177} Although 1, 3-\(\beta\)-glucan is also an important component of the cell wall of \textit{Fusarium} \textit{spp.},\textsuperscript{178} the structure analysis and related immuno reactions of fungal cell wall were mainly done in the species of \textit{Aspergillus famigatus}, \textit{Saccharomyces cerevisiae} and \textit{Candida albicans}.\textsuperscript{167,168,171,179-182}

\textbf{Conclusion and Further Studies Proposed}

\textbf{Conclusion}

In our study, evidence for infection such as increased level of CD8+ T cells and neutrophils was demonstrated in both nasal polyp and chronic sinusitis. A significantly higher number of natural killer cell in nasal polyp tissue also suggests the contribution of innate immunity in the pathogenesis of the disease. Although ImmunoCAP of serum specific IgE to common allergens was not able to show an increased incidence of atopy in nasal polyp and chronic sinusitis patients compared to that in the general population, eosinophilia remained to be the common feature in our study patients. A self-developed Immunodot blot array system proved serum specific IgE to less commonly tested allergens, especially \textit{Trichophyton rubrum} in nasal polyps and chronic sinusitis patients. This finding was further confirmed by IgE western blot. Protein characterization of the allergens revealed homology with a 35 kDa heat shock protein and 1, 3-\(\beta\)-glucanosyltransferase in \textit{Fusarium} \textit{spp.} with unclear biological functions.

Innate immunity is well defined against fungal infection. Although neutrophil and NK
cell infiltration in nasal polyps or inflamed sinus mucosa was evidenced in our study, the loci of the two cells were not universally the same, suggesting that their recruitment may be caused by some other factors than infection alone. CD8+ T cell (cytotoxic T cell) is important in infectious disease. However, commonly identified eosinophil activation in our patients suggested a Th2 polarization. Although chronic sinusitis is mainly characterized by Th2 upregulation, nasal polyp is a disease with a mixed type of Th1 and Th2. Previous studies have reported that *T. rubrum* may initiate Th2 polarization which predisposes to sensitization in patients with chronic *T. rubrum* infections. The discrepancy of sera specific IgE to *T. rubrum*, the lack of langerhans cells and the absence of an increased mast cell level may suggest that *T. rubrum* sensitization occurs through skin or nail rather than airway mucosa. Allergens may also enter the nasal or sinus cavity through systematic absorption besides direct contact. Therefore, whether or not a positive fungal culture in nasal/sinus cavity is necessary for the diagnosis of allergic fungal sinusitis should be the subject of further discussions. The role of *T. rubrum* as a superantigen is also suspected.

Nasal polyp and chronic sinusitis exit chronic inflammation. Although both allergy and infection have been suggested contribute to their development, the on-set of the diseases remains unclarified. It is interesting that evidence of both infection and IgE-mediated allergy, especially to fungi, i.e. *Trichophyton rubrum* have been evidenced by our study. In the study of atopic dermatitis, it has been reported that dermatophytosis caused by *T. rubrum* often shows interplay of fungus and bacteria,
such as *S. aureus, M. sedantarius, B. epidermidis, C. minutissimum, Corynebacterium jeikeium, Pseudomonas* and *Proteus spp.*.\textsuperscript{109} The growth of *T. rubrum* produces a penicillin- and streptomycin-like substance which favors secondary antibiotic-resistant bacteria infections.\textsuperscript{151} On the other side, bacteria will cause tissue damage by secretion of a proteolytic enzymatic substance.\textsuperscript{109} The interaction between dermatophyte and bacteria usually results in a low recovery rate from the related diseases. Some bacteria, such as *M. sedantarius* and *B. epidermidis* are able to produce sulfur compounds which are potent antifungal agents.\textsuperscript{109} In this case, the dermatophyte will be suppressed, and as a consequence, the primary dermatophyte-positive interspaces will become converted into macerated interspaces. Whether the findings in trichophytosis can also be identified in nasal polyp tissue or chronic sinusitis remains to be investigated. An animal model may help us to understand the development of the chronic inflammation in nasal polyp and chronic sinusitis. Follow-up study of patients with trichophytosis but without nasal polyp or chronic sinusitis may provide additional important information of risk factors predisposing initiation as well as development of the diseases.
Further Studies Proposed

1. Immunodeficiency, a common disease in chronic sinusitis and nasal polyps?

Natural immunity has the ability to kill or suppress the growth of the pathogens and limit their spread through cell-mediated immunity. Fungi, especially those mainly causing superficial infections, are usually taken as opportunistic pathogens. Previous
studies have suggested that the incidence of immuno disturbance in chronic sinusitis may be underestimated. Therefore, further studies of systemic examinations of the patients, especially blood tests, may be of great help to understand the predisposing factors of the development of nasal polyps and chronic sinusitis.

2. *T. rubrum*, an inhalant allergen?

The discrepancy between serum specific IgE to *T. rubrum* and lack of langerhans cells or increased mast cell levels may be due to prior sensitization though nail or skin rather than the airway. Therefore, it was strongly suggested that all our nasal polyp and chronic sinusitis patients should be carefully examined by dermatologists with a fungal culture to test the involved species. Further study to raise polyclonal antibodies against the antigens of interest will help us to evaluate the presence of *T. rubrum* in the actual tissues, including nail and skin tissues, and especially nasal polyp or inflamed sinus mucosa. Immunohistochemistry study of diseased skin or nail tissue caused by trichophytosis may help us to understand the development of the *T. rubrum* infection, including the question whether the theory of superantigen is applicable or not.

3. Sti35 and 1, 3-β-glucanosyltransferase, promising antigens for understanding biological functions of *T. rubrum* and vaccine development.

Due to the short amino acids sequence determined by N-terminal sequencing,
homologus information was quite limited in our study. A good match would be one that would have more than one peak matching the sequence of the interest. As discussed in chapter 5.6.2.6, most eukaryotic cells are blocked as a result of posttranslational modification. Deblocking the protein or internal sequencing may provide important homologous information.

The antigenecity of proteins from *T. rubrum* is quite consistent in our study. The function of the heat shock protein and 1, 3-β-glucanosyltransferase, although highly conserved in eukaryotes, are not well characterized. Because of the lack of a database, cDNA clone of the allergens is necessary. In vivo and in vitro tests, such as lymphocyte challenge with crude extract, and recombinant proteins will give important information on immuno reactions caused by sti35 and 1, 3-β-glucanosyltransferase, including and their unique features in *T. rubrum* or common features conserved by other eukaryotes. Also, studies in normal controls may provide important information on different host reactions to the same allergen. In addition, vaccine developed based on the recombinant proteins may be very promising in the treatment of nasal polyps and chronic sinusitis.


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