Prevalence and severity of asthma, rhinoconjunctivitis and eczema in children from the Bangkok area: The Global Asthma Network (GAN) Phase I

Sasawan Chinratanapisit,1 Nariyara Suratannon,2 Punchama Pacharn,3 Paskorn Sritipsukho,4,5 Pakit Vichyanond3

Abstract

Background: As noted in the reports of ISAAC phase I and III, allergic diseases are very common in Thailand, especially among younger children.

Objective: The objectives of this project are to study the prevalence and severity of the most common allergic diseases, i.e. asthma, rhinoconjunctivitis and eczema among children living in Bangkok.

Methods: A cross-sectional multi-centers survey using GAN Core questionnaires on asthma, rhinoconjunctivitis and eczema symptoms were completed by parents of children aged 6–7 years and children aged 13–14 years.

Results: The total of 6,291 questionnaires were eligible for the analysis. The cumulative vs. 12-month period prevalence of the three conditions for all children were: 24.4% vs. 13.5% for wheezing, 51.1% vs. 43.6% for rhinitis and 15.8% vs. 14.2% for eczema, respectively. The period prevalence of wheezing for younger children (14.6%) was higher than for older children (12.5%). Prevalences of severe wheeze and exercise wheeze were more common among older children (2.9% and 14.8%). The 12-month prevalences of rhinitis (43.6%) and rhinoconjunctivitis (16.3%) were higher in both age groups. Eczema, as the same to the other conditions, occurred more frequently in both groups (period prevalence of 14.3% and 14.0%) comparing to ISAAC phase III.

Conclusion: Allergic conditions are very common diseases among children residing in Bangkok. There is an urgent need for an in-depth study to define epidemiological factors responsible for this increase.

Key words Asthma, rhinoconjunctivitis, eczema, ISAAC, GAN

Introduction

Allergic diseases are among the most common chronic diseases in children and adolescents leading to a substantial health and socioeconomic burden. The International Study of Asthma and Allergy in Childhood (ISAAC) phase I and III surveys reported an overall increase in the prevalence of eczema and allergic rhinoconjunctivitis worldwide. However, no changes in the prevalence of asthma among 13-14-year-old children over a mean period of 7 years was observed.1-3

The ISAAC phase I study in Thailand was conducted in 1995-1999 in 3 cities namely; Bangkok,4 Chiang Mai5 and Khon Kaen.6 In Bangkok, the prevalences of three conditions were: asthma 18.3%, rhinitis 44.2% and eczema 15.4%. The ISAAC phase III studying in Bangkok shown that there is a trend of increasing prevalence of all atopic diseases among children.7

The Global Asthma Network (GAN), established in 2012, was formed by scientists from the International Study of Asthma and Allergies in Childhood (ISAAC) 1991–2012 (phases
I,11 II14 and III13,15) and from the International Union Against Tuberculosis and Lung Disease (The Union16–19) following production of the first Global Asthma Report (GAR) 2011,20 launched in New York (NY, USA) in 2011 at the time of the United Nations high-level meeting on non-communicable diseases. GAN phase I, builds on the ISAAC findings by collecting further information on asthma, rhinitis and eczema, prevalence, severity, diagnoses, asthma emergency room visits, hospital admissions, management and the use of asthma essential medicines.

The objectives of our project are to study the prevalence and severity of the most common allergic diseases. i.e. asthma, rhinoconjunctivitis and eczema in children living in Bangkok. We, herein, report the results of our GAN phase I study in 6,291 children from the two age groups living in the Bangkok area.

Methods
Study Design
This study is a cross-sectional, multi-center, study design.

Participants
Seven primary schools and six secondary schools in Bangkok were randomly mapped, stratified and had chosen to represent the population of the entire Bangkok Metropolitan area. In addition, equal numbers of governmental and private schools were selected to avoid an over representation of any predominant socioeconomic classes. Subjects were selected in the same manner as ISAAC phase III. The same age groups were used: 13–14 years old adolescents (self-completed questionnaires) and 6–7 years old children (parental completed questionnaires) and GAN phase I adds their parents as an adult group. Students of both age groups were selected either by grade/level/year or by age group. The questionnaires were sent out to 6,824 children (3,544 for 6–7 years and 3,280 for 13–14 years). Although participation rates for both age groups from these schools were exceptionally high (92.18%), many questionnaires were incompletely answered and were therefore excluded from the analysis. This left a grand total of 6,291 children (3,074 for 6–7 years and 3,217 for 13–14 years) for the inclusion of the analysis. The study was approved by the Human Research Ethics Committee of Thammasat University (054/2560) and the Human Research Ethics Committee of Bhumibol Adulyadej Hospital. The clinical trial number was MTU-EC-ES-4-013/60. Inform consents/assents were obtained by children and by the parents.

GAN Core Questionnaires
GAN Standardized Written Core Questionnaires developed from ISAAC Questionnaires for use in phases I and III, were used in GAN. Demographic questionnaires includes the participant’s name, age, date of birth, school (for the adolescents and children), sex and date of interview. Questionnaires were coded by using a unique number for each center, school and participant to ensure confidentiality and to link the questionnaires between the adults, adolescents and children.21 The written core questionnaires, that was used in ISAAC, have had a question about doctor-diagnosis about asthma, rhinitis and eczema. The core questions were both sensitive and specific, had good content, constructive and concurrent and predictive validity.22 As in ISAAC, a video of asthma questionnaires was an optional tool: the international version that is being used in ISAAC.23 This 6-minute non-verbal video showed the clinical signs of asthma symptoms and was developed by the Wellington Asthma Research Group, in order to avoid the problems of translation and understanding of terms of “wheeze” or “whistling” and their uses in culturally heterogeneous population.24 The video has the advantage of obtaining data from many students quickly and efficiently. The questionnaires were translated into Thai and back translated by a three linguistic proficient individuals and were reviewed and approved by the investigators.

Sample Size
As in ISAAC, a sample size of 3,000 participants per age group (and therefore potentially 6,000 adults of each group) was used. The sample size provided greater than 99% ability (at the 1% level of significance) to detect differences in the prevalence of wheezing of 30% in one center and 25% in another center.22 As sampling was done by schools, and the information gained from the school pupils and adults, is likely to be a cluster effect. Like ISAAC, the analysis incorporated adjustments in cluster sampling using the design effect,25 which is important for large studies where clusters of different sizes may be used in different regions. High participation is sought for GAN phase I: at least 80% for 13–14 years old and 70% for 6–7 years old and 70% for adults/parents.

Data Collection and Analysis
Data were collected from July 2017 up to February 2018. Information on the questionnaires was entered in the GAN Epi-Info data entry packaged by GAN Global Center in Auckland, New Zealand (info@globalasthmanetwork.org). Such data were analyzed by using STATA version 14 and expressed in the prevalence of three diseases in both the younger and older groups, separately.

Results
Positive response to wheezing modules for younger and older age groups as well as for all children surveyed are tabulated in Table 1. All participants are Thai. The prevalence of ever-wheeze in the younger age group was slightly higher than in the older age group (26.0% vs. 22.9%, p = 0.004). This was also true for percentage of current wheeze or wheeze in the past 12 months (14.6% vs. 12.5%, p = 0.016) and for attacks within the past 12 months (14.4% vs. 12.6%, p = 0.029). Percentages for severe wheeze (1.9% vs. 2.9%, p = 0.019) and exercise wheeze (3.0% vs. 14.8%, p < 0.001) were much higher among older children. Percentages of night awakening were slightly higher among the younger age group (6.7% vs. 4.2%, p < 0.001). Percentages of night cough were noticeably high in both groups (24.2% and 29.9%, p < 0.001). The prevalence for diagnosed asthma (asthma-ever, 6.1% and 8.8%, p < 0.001) were much lower than wheezing-ever for both groups (26.0% and 22.9%). As for male: female ratio, there was no predominance for males over females other than responses for question of ‘asthma ever’ (1.36).
Table 1. Percent of positive response of questions in wheezing module.

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>All (n = 6,291)</th>
<th>6-7 years (n = 3,074)</th>
<th>13-14 years (n = 3,217)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Current wheeze</td>
<td>13.5 (12.7, 14.3)</td>
<td>14.6 (13.4, 15.9)</td>
<td>12.5 (11.4, 13.7)</td>
<td>0.016</td>
</tr>
<tr>
<td>Wheezing ever</td>
<td>24.4 (23.4, 25.5)</td>
<td>26.0 (24.5, 27.6)</td>
<td>22.9 (21.5, 24.4)</td>
<td>0.004</td>
</tr>
<tr>
<td>Asthma ever</td>
<td>7.4 (6.8, 8.1)</td>
<td>6.1 (5.2, 6.9)</td>
<td>8.8 (7.8, 9.7)</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Symptoms in past 12 months

- attacks                     | 13.5 (12.6, 14.3) | 14.4 (13.2, 15.7) | 12.6 (11.4, 13.7) | 0.029   |
- night waking                | 5.4 (4.9, 6.0) | 5.4 (4.9, 6.0) | 4.2 (3.5, 4.9) | < 0.001 |
- severe wheeze               | 2.4 (2.0, 2.8) | 2.4 (2.0, 2.8) | 2.9 (2.3, 3.4) | 0.019   |
- exercise wheeze             | 9.0 (8.3, 9.8) | 9.0 (8.3, 9.8) | 14.8 (13.6, 16.0) | < 0.001 |
- night cough                 | 27.1 (26.0, 28.2) | 27.1 (26.0, 28.2) | 29.9 (28.3, 31.5) | < 0.001 |

Table 2. Percent of positive response to video questionnaires for wheezing

<table>
<thead>
<tr>
<th>Description of video sequences:</th>
<th>13-14 years (n = 3,217)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cumulative (95%CI)</td>
</tr>
<tr>
<td>Wheezing at rest</td>
<td>11.9 (10.8, 13.1)</td>
</tr>
<tr>
<td>Exercise wheeze</td>
<td>13.5 (12.3, 14.5)</td>
</tr>
<tr>
<td>Night wheeze</td>
<td>6.6 (5.8, 7.5)</td>
</tr>
<tr>
<td>Night cough</td>
<td>23.4 (21.9, 24.8)</td>
</tr>
<tr>
<td>Severe wheeze</td>
<td>8.1 (7.2, 9.1)</td>
</tr>
</tbody>
</table>

Table 3. Percent of positive response of questions in rhinitis modules.

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>All (n = 6,291)</th>
<th>6-7 years (n = 3,074)</th>
<th>13-14 years (n = 3,217)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Current rhinoconjunctivitis or Current AR</td>
<td>16.3 (15.4, 17.2)</td>
<td>15 (13.8, 16.3)</td>
<td>17.5 (16.2, 18.8)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Current nose symptom</td>
<td>43.6 (42.4, 44.8)</td>
<td>38.2 (36.5, 39.9)</td>
<td>48.8 (47.0, 50.5)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Current eye symptom</td>
<td>16.6 (15.6, 17.5)</td>
<td>15.0 (13.8, 16.3)</td>
<td>18.0 (16.7, 19.4)</td>
<td>0.001</td>
</tr>
<tr>
<td>Nose ever</td>
<td>51.1 (49.9, 52.4)</td>
<td>47.3 (45.5, 49.0)</td>
<td>54.9 (53.1, 56.6)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Hay fever ever</td>
<td>27.4 (26.3, 28.5)</td>
<td>24.5 (23.0, 26.0)</td>
<td>30.1 (28.5, 31.7)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Severe rhinoconjunctivitis</td>
<td>1.5 (1.2, 1.7)</td>
<td>1.0 (0.6, 1.3)</td>
<td>1.9 (1.4, 2.4)</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

The self-reported video questionnaires completing by the 13-14-year-old group revealed a cumulative vs. current prevalence of: wheezing at rest (11.9% vs. 8.9%), exercise wheeze (13.5% vs. 9.0%), night wheeze (6.6% vs. 5.6%), night cough (23.4% vs. 17.9%) and severe wheeze (8.1% vs. 5.8%) (Table 2). Percentages for night wheeze (5.6%) was slightly higher than that derived from the written questionnaires (4.2%). The video responses to exercise question (9.0%) was lower than that from the written ones (14.8%). The prevalence of severe wheeze from video responses was 5.8%, which is twice of the written questionnaire (2.9%).

In Table 3, prevalences of rhinitis and other associated symptoms are shown. An exceptionally high number of children from both age groups (47.3% and 54.9%) reported nasal...
The cumulative prevalence of wheezing based on the video questionnaires from this study (11.9%) is closed to the prevalence of the ISAAC study phase III from Bangkok (11.5%). This is much higher than the Asia-Pacific prevalence (5.5%) and, also the global prevalence (8.7%) of the ISAAC study phase III. The prevalence of severe asthma (written questionnaires) in the 13-14 years age group is 2.9%. This is lower than the prevalence of severe asthma from the ISAAC study phase III: globally (6.9%) ranging from 3.8% in Asia-Pacific, Northern and Eastern Europe to 11.3% in North America (compared to Bangkok 4.0%).

The Asthma Insight and Management (AIM) survey (2011) reported the asthma exacerbations in the past 12 months: Thailand (36%), South Korea (47%), Australia (54%), and China (67%). Thai patients that uses controller medication is 54% in previous month. Pill controller medication is the most common form among those reporting controller medication used (67%), whereas 57% reported taking an inhaler.

The new GAN phase I survey, however, portrayed a differing epidemiological outlook than from what has been felt among practitioner caring for asthmatic patients. These preliminary data have shown that prevalence of asthma in younger and older children is still over 10% of the population surveyed. Moreover, the prevalence for those with severe wheeze is roughly 2%. The Chest and Allergy Societies in Thailand have regularly updated asthma guidelines for adults and children. Besides, social media has made it easier for parents/patients to find appropriate professional care. An increase in the availability of asthma controllers throughout the country may help lessen the severe asthma attacks presented to emergency rooms and requiring hospital admissions in this country. Among these drugs, inhaled steroids are very popular. Since generic versions of these controllers are cheaper than original version, they were included in Essential Drug List subsidized by the Government for those eligible for medical supports (governmental employees, those under the social security program and universal health coverage). Effective advocacy by non-governmental organizations, smoking in homes and public places is now rare event. Thailand has enforced stricter regulations to reduce outdoor air pollution, such as cleaner air emissions and vehicle fuels.

Table 4. Percent of positive response of questions in eczema module.

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>All (n = 6,291) (95%CI)</th>
<th>6-7 years (n = 3,074) (95%CI)</th>
<th>13-14 years (n = 3,217) (95%CI)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rash ever</td>
<td>15.8 (14.9, 16.7)</td>
<td>16.3 (15.0, 17.6)</td>
<td>15.2 (14.0, 16.5)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Eczema ever</td>
<td>22.8 (21.8, 23.9)</td>
<td>28.6 (27.0, 30.2)</td>
<td>17.3 (16.0, 18.7)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Flexural area</td>
<td>10.8 (10.1, 11.6)</td>
<td>11.7 (10.6, 12.9)</td>
<td>10.0 (8.9, 11.0)</td>
<td>0.024</td>
</tr>
<tr>
<td>Symptoms in past 12 months</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- rash</td>
<td>14.2 (13.3, 15.0)</td>
<td>14.3 (13.1, 15.6)</td>
<td>14.0 (12.8, 15.2)</td>
<td>0.684</td>
</tr>
<tr>
<td>- rash clear</td>
<td>9.6 (8.9, 10.3)</td>
<td>9.1 (8.1, 10.2)</td>
<td>10.0 (9.0, 11.1)</td>
<td>0.226</td>
</tr>
<tr>
<td>- night waking</td>
<td>4.7 (4.2, 5.2)</td>
<td>5.6 (4.8, 6.4)</td>
<td>3.8 (3.2, 4.5)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Severe eczema: Current eczema associated with sleep disturbance 1 or more nights per week
P Value for Chi square test of positive response symptom between age groups

Discussion

As noted in the reports of ISAAC phase I and III, asthma was very common in Thailand, especially among younger children. In this study, prevalence rates of current wheeze based on the written questionnaire in the 6–7 years is similar to the prevalence in the ISAAC study phase III; in Bangkok (14.6% vs. 15.0%, p = 0.541). Meanwhile, the prevalence rate in the 13–14 years age group is slightly lower than prevalence in the ISAAC study phase III; in Bangkok (12.5% vs. 13.9%, p = 0.024). Slightly higher than the ISAAC phase III: the mean global prevalence for current wheeze (11.5% and 4.9%) and the Asia-pacific prevalence (9.5% and 8.8%).
Ecological economic analyses also revealed that although the high-income centers tended to have a higher prevalence of current wheeze, a reverse trend was found in the prevalence of symptoms of severe asthma among current wheezers. There may be several reasons underlying this observation. First, asthma care is likely to be poorer in these developing countries, although a recent epidemiological survey showed that suboptimal asthma management was a global phenomenon. Secondly, there may be less awareness of wheeze being a symptom of asthma, even in those with frequent wheezing, similar to the situation amongst ethnic minorities in developed countries. This notion is further supported by the finding that undiagnosed asthma among those current wheezers with severe asthma symptoms was most commonly seen in these lower income countries. Children with undiagnosed frequent symptoms are also more likely to receive inadequate care for their asthma and may fall into a vicious downward spiral of asthma control. Thirdly, differences in the levels of environmental exposure, including air pollutants and infective agents, may also contribute to the greater severity observed in these countries.

GAN phase I has provided the most comprehensive estimate of the worldwide symptom prevalence of asthma to date. This global map of asthma is invaluable not only for public health planning, but also for generating hypotheses in explaining the etiological factors for this common disorder.

In our study, the prevalence of current AR or current rhinoconjunctivitis in the 6–7 year and 13–14-year age groups are 15.0%, 17.5% respectively. As the ISAAC study phase III, the prevalence of current AR of Thai children from the Bangkok area were 13.4% and 23.9% respectively. It is slightly higher than the mean of global prevalence (9.1%, 16%), and the Asia-Pacific prevalence (5.8% and 14.5%).

In our study, the prevalence of current eczema symptoms in the 6–7 years and 13–14 years age groups are 14.3%, 14.0% respectively. These values are slightly higher than those from the ISAAC study phase III study in Bangkok (13.3% and 10.4%). However, our GAN results on eczema is much higher than the ISAAC study phase III study elsewhere: the mean global prevalence (7.9%, 7.3%) and the Asia-Pacific prevalence (4.7% and 5.3%).

For developing countries, Thailand has been noted to have an increase in the number of patients with food allergy and atopic dermatitis. The reason for this worrisome and unusual increase is uncertain at this point. Similarly, results of GAN phase I survey substantiate the increasing numbers of children in both age groups. If a phenomenon of allergic march operates in this part of the world, one should witness an increase in the number of asthmatic patients rather than a decrease in the next decade.

### Strengths and Weaknesses of the Study

The major strengths of our study included a standardized written core questionnaires (GAN 2016) developed from ISAAC Questionnaires, well-established standardized protocol and high response rate. The establishment of GAN 2016 questionnaires allows an excellent opportunity for different countries to establish their own basic epidemiological data for allergic diseases that can be compared internationally. A video asthma questionnaire (6-min non-verbal video) shows clinical signs of asthma symptoms to avoid problems of translation and comprehension of terms such as “wheeze” or “whistling” and their use in culturally heterogeneous population. One limitation of our study is that symptoms of allergic rhinitis were self-reported in the questionnaire, therefore, we could not confirm with physical examination and laboratory investigations.

In conclusion, the result of GAN phase I in Bangkok showed a slightly increase of prevalence of eczema in both age groups, while prevalences of asthma and allergic rhinitis have become stabilized in both age groups. Most Thai children with asthma had coexisting rhinitis, and a portion of patients with rhinitis also had asthma. Allergic conditions are very common among children residing in Bangkok. There is an urgent need for an in-depth study to define epidemiological factors responsible for this increase.

### Acknowledgements

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The authors would like to thank all the children, parents, and teachers who participated in this study. We also thank those who helped with field works.

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### References


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ORIGINAL ARTICLE

Prevalence and risk factors of allergic rhinitis in children in Bangkok area

Sasawan Chinratanapisit,1 Narissara Suratannon,2 Punchama Pacharn,3 Paskorn Sritipsukho,4,5 Pakit Vichyanond6

Abstract

Background: Allergic rhinitis (AR) is a disease with a high global disease burden and significant morbidity and expense. Risk factors are not well understood.

Objective: The objective of our project is to study the prevalence and risk factors of AR in children living in the Bangkok area.

Methods: A cross-sectional, multi-center survey using new GAN core questionnaires on current AR and risk factors was completed by 3,074 parents of children aged 6–7 years and by 3,217 children aged 13–14 years, directly.

Results: The prevalence of current AR in children aged 6–7 years and 13–14 years was 15.0% (95% confidence interval [CI]:13.8–16.3%) and 17.5% (95% CI: 16.2–18.8%), respectively. The prevalence of severe AR in children aged 6–7 years and 13–14 years was 1.0% (95% CI: 0.6–1.3%) and 1.9% (95% CI: 1.4–2.4%), respectively. Co-morbidity with asthma and eczema was 27.1% and 24.6%, respectively. Significant factors associated with AR include parental history of asthma (p = 0.025), parental history of AR (p < 0.001), parental history of eczema (p < 0.001), lower respiratory tract infection in the first year of life (p < 0.001), breastfeeding (p = 0.019), current use of paracetamol (p < 0.001), exercise (p < 0.001), current cat exposure (p = 0.008), and truck traffic on the street of residence (< 0.001).

Conclusion: AR is a common disease among children residing in Bangkok. This study confirms that a family history of atopy (asthma, AR, and eczema), antibiotics given in the first year of life, current paracetamol use, exercise, current cat exposure, and truck traffic on the street of residence are important and significant risk factors for AR symptoms.

Key words: allergic rhinitis, atopy, asthma, ISAAC, GAN

Introduction

Allergic rhinitis (AR) is characterized by paroxysms of sneezing, rhinorrhea, and nasal obstruction, often accompanied by itching of the eyes, nose, and palate. Postnasal drip, cough, irritability, and fatigue are other common symptoms.1,2 AR is associated with significant morbidity and expense.3,4

The increase in the prevalence of AR began to attract attention from epidemiologists in the late 1980s. The International Study of Asthma and Allergies in Childhood (ISAAC) was initiated to establish the prevalence of allergic diseases in 257,800 school children aged 6–7 years and in 463,801 children aged 13–14 years using standardized and validated questionnaires.7 Phase I of ISAAC, which began to enroll patients in 1992, sought to establish prevalence rates in nearly 60 countries on every continent; phase II investigated variables contributing to AR (e.g., environmental exposures); and phase III provided follow-up data on the patients at least five years after entry into the study. In phase I, prevalence rates for AR collected across all centers ranged from 0.8% to 14.9% (median, 6.9%)
in the 6–7-year-olds and from 1.4% to 39.7% (median, 13.6%) in the 13–14-year-olds. The highest prevalence rates for AR were observed in parts of Western Europe, North America, and Australia, whereas the lowest rates were found in parts of Eastern Europe and South and Central Asia. The phase III analyses revealed that the prevalence rates had increased, with 12-month prevalence rates of 1.8% to 24.2% in children aged 6–7 years (median, 8.5%) and 1.0% to 45% (median, 14.6%) in children aged 13–14 years. These findings strongly indicate that the prevalence of AR has increased over a relatively short period of time, mostly in Westernized countries with a higher standard of living.

According to phase I of ISAAC in Bangkok (1995–1999), the prevalence of AR was 10.0% in the children aged 6–7 years and 15.4% in the children aged 13–14 years. In phase III of the study in Bangkok (2001), the prevalence of AR in children aged 6–7 years and 13–14 years was 13.4% and 23.9%, respectively. There was an increase in the prevalence of rhinitis in both age groups.

Phase III of ISAAC included new questions on risk factors that identified several environmental associations. Risk factors for AR include paracetamol, antibiotics, truck traffic, breastfeeding, farm animals, cats and dogs, air pollution, tobacco, body mass index (BMI), diet, cooking fuels, birth weight, migration, and siblings. Despite the considerable research efforts, the risk factors of AR remain poorly understood. A family history of atopic diseases seems to be a major risk factor, but various environmental factors and lifestyle are also considered important elements in the evolution of the disease.

The objective of our project is to study the prevalence and risk factors of AR in children living in Bangkok, Thailand.

Methods

Study Design

This study has a cross-sectional, multi-center design.

Participants

Seven primary schools and six secondary schools in Bangkok were randomly mapped, stratified, and chosen to represent the population of the entire Bangkok metropolitan area. Subjects were selected in the same manner as ISAAC phase III. The same age groups were recruited: 13–14-year-old children (self-completed questionnaires) and 6–7-year-old children (parental completed questionnaires). Of 6,834 questionnaires sent to children, 6,291 were completed (95.05%). There were 3,074 (86.49%) questionnaires of children aged 6–7 years and 3,217 (98.08%) questionnaires of children aged 13–14 years available for analysis. The study was approved by the Human Research Ethics Committee of Thammasat University (054/2560) and the Human Research Ethics Committee of Bhumibol Adulyadej Hospital. The clinical trial number was MTU-EC-ES-4-013/60. Informed consents/assents were obtained from the children and parents.

GAN Care Questionnaires

GAN 2016 standardized written core questionnaires for AR modifying from ISAAC questionnaires were used in this study. The questionnaires were translated and back-translated into the Thai language by three independent linguistic proficient individuals. Demographic questions included the participant’s name, age, date of birth, school (for the adolescents and children), sex, and date of interview. Questionnaires were coded by using a unique number for each center, school, and participant to ensure confidentiality and to link the questionnaires between the adults and children. The written core questionnaires, used in GAN, had a question about doctor-diagnosed asthma, rhinitis, and eczema added. The core questions were both sensitive and specific, and they had good content, construct, concurrent, and predictive validity. The environmental risk factor questionnaires, developed for ISAAC phase III, were expanded for use in this study. Height and weight measurements were taken by the fieldworkers in schools.

Definitions of AR, Rhinitis, and Hay Fever

The standardized core symptom questionnaire was the same as that used in ISAAC phase I and comprised of six questions on symptoms relating to rhinitis or rhinoconjunctivitis. These questions were as follows:

1. Have you (has your child) ever had a problem with sneezing or a runny or blocked nose when you (he or she) DID NOT have a cold or “the flu”?
2. In the past 12 months, have you (has your child) had a problem with sneezing or a runny or blocked nose when you (he or she) DID NOT have a cold or “the flu”?
3. In the past 12 months, has this nose problem been accompanied by itchy/watery eyes?
4. In which of the past 12 months did this nose problem occur? (Month names listed)
5. In the past 12 months, how much did this nose problem interfere with your (child’s) daily activities? (Not at all, a little, a moderate amount, a lot)
6. Have you (has your child) ever had hay fever?

Question 2 was used to estimate the prevalence of current rhinitis; question 3 was used to estimate the prevalence of current conjunctivitis; and question 6 was used to estimate the prevalence of “hay fever ever.” Questions 2 and 3 were combined to assess current rhinoconjunctivitis symptoms or current AR. Questions 2 and 3 and the answer “A LOT” to question 5 were used to assess the prevalence of severe rhinoconjunctivitis symptoms or severe AR.

Sample Size

A sample size of 2,654 is needed to estimate the prevalence of questionnaire-based AR of 10% for children of each age group with margin errors of ±1.5% and type one error of 0.01. The total sample size of 6,834 was accounted for the non-response rate of 30%.

Data Collection and Analysis

Data were collected from July 2017 to February 2018. Statistical analyses were carried out using STATA/SE software (Stata/SE 14 for Windows, StataCorp LP, College Station, TX, USA). Binomial confidence intervals (CIs) on proportions with rhinitis and rhinoconjunctivitis were calculated. The multivariable logistic regression model was used to conduct exploratory analysis for risk factors of AR. The model included...
age, sex, family history of allergy, birth weight, paracetamol, antibiotics, truck traffic, breastfeeding, farm animals, cat and dog exposure, air pollution, tobacco, BMI, diet, cooking fuels, migration, and number of older and younger siblings to estimate the magnitude of the association by calculating adjusted odds ratios with their 95% CIs.

Results

The prevalence of questionnaire-based symptoms of rhinitis stratified by age group is shown in Table 1. The prevalence of current rhinitis in children aged 6–7 years and 13–14 years was 38.2% (95%CI: 36.5–39.9%) and 48.8% (95%CI: 47.0–50.5%), respectively. The prevalence of current rhinitis in all children was 43.6% (95%CI: 42.4–44.8%). Concomitant eye symptoms were reported at 16.3%. The prevalence of current AR in children aged 6–7 years and 13–14 years was 15.0% (95%CI: 13.8–16.3%) and 17.5% (95%CI: 16.2–18.8%), respectively. The prevalence of current AR in all children was 16.3% (95%CI: 15.4–17.2%).

Although the term so-called “hay fever” does not exist in the Thai language, 27.4% indicated that they suffered from “allergy to the air,” a common term denoting hay fever in Thailand.

Patterns of rhinitis symptoms of children in Bangkok were of the perennial type. The prevalence of severe AR in children aged 6–7 years and 13–14 years was 1.0% (95%CI: 0.6–1.3%) and 1.9% (95%CI: 1.4–2.4%), respectively. The prevalence of severe AR in all children was 1.5% (95%CI: 1.2–1.7%). There were strong associations with other allergic diseases: 27.1% of children with AR had asthma and 24.6% had eczema.

A parental history of atopy including asthma (p = 0.025, OR = 1.50, 95%CI = 1.05–2.13), AR (p < 0.001, OR = 1.43, 95%CI = 1.10–1.71), and eczema (p < 0.01, OR = 1.56, 95%CI = 1.29–1.88) was significantly related to current AR. Current use of paracetamol was associated with current AR (p < 0.001, OR = 1.64, 95%CI = 1.30–2.08). Exercise was associated with current AR (p < 0.001, OR = 1.49, 95%CI = 1.29–1.71). Only current cat exposure was associated with current AR (p = 0.008, OR = 1.28, 95%CI = 1.07–1.54). The frequency of truck traffic on the street of residence was positively associated with current AR; comparison of both the occasional truck traffic group (p = 0.002, OR = 1.28, 95%CI = 1.10–1.50) and the always truck traffic group (p < 0.001, OR = 1.73, 95%CI = 1.41–2.11) to the never truck traffic group is shown in Tables 2 and 3.

Table 1. Prevalence of questionnaires-based symptoms of rhinitis stratified by age group

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>All (n = 6,291)</th>
<th>6-7 years (n = 3,074)</th>
<th>13-14 years (n = 3,217)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Prevalence 95% CI</td>
<td>N</td>
</tr>
<tr>
<td>Current AR or ARC</td>
<td>1,042</td>
<td>16.3% (15.4%, 17.2%)</td>
<td>462</td>
</tr>
<tr>
<td>Current rhinitis</td>
<td>2,744</td>
<td>43.6% (42.4%, 44.8%)</td>
<td>1,175</td>
</tr>
<tr>
<td>Hay fever (allergic to air)</td>
<td>1,722</td>
<td>27.4% (26.3%, 28.5%)</td>
<td>754</td>
</tr>
<tr>
<td>Severe AR</td>
<td>91</td>
<td>1.5% (1.2%, 1.7%)</td>
<td>30</td>
</tr>
</tbody>
</table>

Current AR or Allergic rhinoconjunctivitis (ARC)- positive to question number 2 and 3
Current rhinitis - positive to question number 2
Hay fever ever- positive to question number 6
Severe AR - positive to question number 2 and 3 and the answer “A LOT” to question 5

Table 2. Characteristics of children with AR stratified by age group

<table>
<thead>
<tr>
<th>Factors</th>
<th>Total (n = 6,291)</th>
<th>6-7 Years old (n = 3,074)</th>
<th>13-14 Years old (n = 3,217)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>n (%)</td>
<td>P-value</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-7</td>
<td>3,074</td>
<td>462 (15.0)</td>
<td>-</td>
</tr>
<tr>
<td>13-14</td>
<td>3,217</td>
<td>562 (17.5)</td>
<td>-</td>
</tr>
<tr>
<td>Sex</td>
<td>0.143</td>
<td>0.023</td>
<td>0.760</td>
</tr>
<tr>
<td>Female</td>
<td>3,013</td>
<td>468 (15.6)</td>
<td>1,559</td>
</tr>
<tr>
<td>Male</td>
<td>3,278</td>
<td>555 (16.9)</td>
<td>1,515</td>
</tr>
<tr>
<td>BMI</td>
<td>0.137</td>
<td>0.172</td>
<td>0.445</td>
</tr>
<tr>
<td>&lt; P85</td>
<td>5,360</td>
<td>857 (16.0)</td>
<td>2,619</td>
</tr>
<tr>
<td>≥ P85</td>
<td>931</td>
<td>167 (17.9)</td>
<td>455</td>
</tr>
</tbody>
</table>
Table 2. (Continued)

<table>
<thead>
<tr>
<th>Factors</th>
<th>Total (n = 6,291)</th>
<th>6-7 Years old (n = 3,074)</th>
<th>13-14 Years old (n = 3,217)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>n (%)</td>
<td>P-value</td>
</tr>
<tr>
<td>Paternal allergy history</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asthma</td>
<td>No</td>
<td>6,107 (976 (16.0))</td>
<td>&lt; 0.001</td>
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<tr>
<td></td>
<td>Yes</td>
<td>184 (48 (26.1))</td>
<td>109 (28 (25.7))</td>
</tr>
<tr>
<td>AR</td>
<td>No</td>
<td>5,234 (775 (14.8))</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>1,057 (249 (23.6))</td>
<td>632 (159 (25.2))</td>
</tr>
<tr>
<td>Atopic</td>
<td>No</td>
<td>5,434 (811 (14.9))</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>857 (213 (24.9))</td>
<td>479 (131 (27.3))</td>
</tr>
<tr>
<td>Sibling</td>
<td>No</td>
<td>2,013 (327 (16.2))</td>
<td>0.961</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>4,278 (697 (16.3))</td>
<td>2,040 (322 (15.8))</td>
</tr>
<tr>
<td>Only 6-7 Years old</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>LBW</td>
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<td>-</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Breast Feeding (6 months)</td>
<td>No</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>-</td>
<td>1,810 (246 (13.6))</td>
</tr>
<tr>
<td>Antibiotics (first 1 year)</td>
<td>No</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>-</td>
<td>1,264 (216 (17.1))</td>
</tr>
<tr>
<td>Paracetamol (first 1 year)</td>
<td>No</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>-</td>
<td>1,138 (237 (20.8))</td>
</tr>
<tr>
<td>LRTI (first 1 year)</td>
<td>No</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>-</td>
<td>1,975 (324 (70.1))</td>
</tr>
<tr>
<td>Farm animal</td>
<td>No</td>
<td>-</td>
<td>2,962 (435 (14.7%))</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>-</td>
<td>112 (27 (24.1))</td>
</tr>
<tr>
<td>Paracetamol</td>
<td>No</td>
<td>893 (99 (11.1))</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>5,398 (925 (17.1))</td>
<td>2,659 (422 (15.9))</td>
</tr>
<tr>
<td>Exercise</td>
<td>No</td>
<td>4,032 (558 (13.8))</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>2,259 (466 (20.6))</td>
<td>810 (154 (19.0))</td>
</tr>
<tr>
<td>Parent Smoke</td>
<td>No</td>
<td>6,025 (982 (16.3%))</td>
<td>0.826</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>266 (42 (15.8))</td>
<td>147 (24 (16.3))</td>
</tr>
<tr>
<td>Pet</td>
<td>Dog Now</td>
<td>No</td>
<td>4,275 (728 (15.0))</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Yes</td>
<td>1,566 (283 (18.1))</td>
</tr>
<tr>
<td>Cat Now</td>
<td>No</td>
<td>5,317 (813 (15.5%))</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>974 (197 (20.2))</td>
<td>315 (54 (17.1))</td>
</tr>
<tr>
<td>Truck Traffic</td>
<td></td>
<td>&lt; 0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Never</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sometime</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Always</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fire Cooking</td>
<td>No</td>
<td>6,036 (979 (16.2%))</td>
<td>0.545</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>255 (45 (17.6))</td>
<td>146 (20 (13.7))</td>
</tr>
<tr>
<td>Env Factors</td>
<td>Cockroach</td>
<td>No</td>
<td>4,273 (664 (15.5%))</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Yes</td>
<td>2,018 (360 (17.8))</td>
</tr>
<tr>
<td>Air Conditioner</td>
<td>No</td>
<td>3,993 (619 (15.5%))</td>
<td>0.028</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>2,298 (405 (17.6))</td>
<td>1,254 (203 (16.2))</td>
</tr>
<tr>
<td>Tree or Flower</td>
<td>No</td>
<td>2,238 (343 (15.3))</td>
<td>0.129</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>4,053 (681 (16.8))</td>
<td>2,278 (356 (15.6))</td>
</tr>
<tr>
<td>Perfume</td>
<td>No</td>
<td>3,591 (557 (15.5%))</td>
<td>0.058</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>2,700 (467 (17.3))</td>
<td>1,538 (263 (17.1))</td>
</tr>
<tr>
<td>School Type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Public</td>
<td></td>
<td>4,170 (671 (16.1))</td>
<td>1,957 (125 (10.5))</td>
</tr>
<tr>
<td>Private</td>
<td></td>
<td>2,121 (353 (16.6))</td>
<td>1,117 (165 (14.8))</td>
</tr>
</tbody>
</table>

Prevalence and risk factors of allergic rhinitis in children in the Bangkok area
Table 3. Factor Associate with AR of all children

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>6-7</th>
<th>13-14</th>
<th>6-7</th>
<th>13-14</th>
<th>6-7</th>
<th>13-14</th>
<th>6-7</th>
<th>13-14</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All</td>
<td>Point (95% CI)</td>
<td>Value</td>
<td>Point (95% CI)</td>
<td>Value</td>
<td>Crude Odds Ratio</td>
<td>Adjusted Odds Ratio</td>
<td>Crude Odds Ratio</td>
</tr>
<tr>
<td>Crude Odds Ratio</td>
<td>1.26 (0.97, 1.66)</td>
<td>0.023</td>
<td>1.21 (0.98, 1.48)</td>
<td>0.084</td>
<td>0.97 (0.81, 1.17)</td>
<td>0.70</td>
<td>1.29 (1.03, 1.61)</td>
<td>0.027</td>
</tr>
<tr>
<td>Adjusted Odds Ratio</td>
<td>1.11 (0.96, 1.29)</td>
<td>0.35</td>
<td>1.11 (0.96, 1.29)</td>
<td>0.35</td>
<td>1.20 (1.05, 1.37)</td>
<td>0.009</td>
<td>1.13 (1.00, 1.29)</td>
<td>0.048</td>
</tr>
<tr>
<td>Sex Male</td>
<td>1.11 (0.97, 1.26)</td>
<td>0.13</td>
<td>1.24 (1.03, 1.49)</td>
<td>0.032</td>
<td>1.24 (1.03, 1.49)</td>
<td>0.032</td>
<td>1.31 (1.07, 1.59)</td>
<td>0.006</td>
</tr>
<tr>
<td>Paternal allergy history</td>
<td>1.28 (1.07, 1.55)</td>
<td>0.005</td>
<td>1.20 (1.03, 1.40)</td>
<td>0.042</td>
<td>1.31 (1.07, 1.55)</td>
<td>0.005</td>
<td>1.31 (1.07, 1.55)</td>
<td>0.005</td>
</tr>
<tr>
<td>Asthma</td>
<td>1.96 (1.61, 2.38)</td>
<td>&lt;0.001</td>
<td>1.58 (1.26, 1.98)</td>
<td>0.001</td>
<td>2.02 (1.64, 2.46)</td>
<td>&lt;0.001</td>
<td>1.77 (1.39, 2.27)</td>
<td>0.001</td>
</tr>
<tr>
<td>Atopy</td>
<td>1.74 (1.50, 1.99)</td>
<td>&lt;0.001</td>
<td>1.41 (1.19, 1.66)</td>
<td>&lt;0.001</td>
<td>1.86 (1.53, 2.25)</td>
<td>&lt;0.001</td>
<td>1.65 (1.32, 2.05)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Allergens</td>
<td>1.18 (1.02, 1.36)</td>
<td>0.007</td>
<td>1.12 (0.96, 1.30)</td>
<td>0.10</td>
<td>1.30 (1.06, 1.60)</td>
<td>0.014</td>
<td>1.25 (1.01, 1.54)</td>
<td>0.032</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>1.52 (1.27, 1.82)</td>
<td>&lt;0.001</td>
<td>1.22 (0.98, 1.51)</td>
<td>0.10</td>
<td>2.01 (1.60, 2.51)</td>
<td>&lt;0.001</td>
<td>1.74 (1.35, 2.25)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Paracetamol</td>
<td>1.16 (1.02, 1.32)</td>
<td>0.037</td>
<td>1.10 (0.96, 1.27)</td>
<td>0.10</td>
<td>1.70 (1.40, 2.05)</td>
<td>&lt;0.001</td>
<td>1.45 (1.17, 1.78)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LRTI</td>
<td>1.53 (1.26, 1.86)</td>
<td>&lt;0.001</td>
<td>1.22 (0.98, 1.51)</td>
<td>0.10</td>
<td>2.01 (1.60, 2.51)</td>
<td>&lt;0.001</td>
<td>1.74 (1.35, 2.25)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Farm animal</td>
<td>1.18 (1.02, 1.36)</td>
<td>0.007</td>
<td>1.12 (0.96, 1.30)</td>
<td>0.10</td>
<td>2.01 (1.60, 2.51)</td>
<td>&lt;0.001</td>
<td>1.74 (1.35, 2.25)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Breast feeding</td>
<td>1.16 (1.02, 1.32)</td>
<td>0.037</td>
<td>1.10 (0.96, 1.27)</td>
<td>0.10</td>
<td>1.70 (1.40, 2.05)</td>
<td>&lt;0.001</td>
<td>1.74 (1.35, 2.25)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Truck traffic</td>
<td>1.56 (1.33, 1.83)</td>
<td>&lt;0.001</td>
<td>1.22 (0.98, 1.51)</td>
<td>0.10</td>
<td>2.01 (1.60, 2.51)</td>
<td>&lt;0.001</td>
<td>1.74 (1.35, 2.25)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cockroaches</td>
<td>1.23 (1.03, 1.47)</td>
<td>0.007</td>
<td>1.12 (0.96, 1.30)</td>
<td>0.10</td>
<td>2.01 (1.60, 2.51)</td>
<td>&lt;0.001</td>
<td>1.74 (1.35, 2.25)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Air conditioner</td>
<td>1.17 (1.02, 1.34)</td>
<td>0.028</td>
<td>1.10 (0.96, 1.27)</td>
<td>0.10</td>
<td>1.70 (1.40, 2.05)</td>
<td>&lt;0.001</td>
<td>1.74 (1.35, 2.25)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Perfume</td>
<td>1.14 (1.00, 1.30)</td>
<td>0.058</td>
<td>1.07 (0.93, 1.23)</td>
<td>0.371</td>
<td>1.21 (0.95, 1.52)</td>
<td>0.106</td>
<td>1.14 (0.95, 1.37)</td>
<td>0.24</td>
</tr>
</tbody>
</table>

* Multivariable logistic regression model
Concerning the age group of 6–7 years, parental history of AR and eczema was significantly related to current AR (AR: p < 0.001, OR = 1.71, 95%CI = 1.35–2.17; eczema: p < 0.001, OR = 1.83, 95%CI = 1.42–2.35). Lower respiratory tract infection (LRTI) in the first year of life was positively associated with current AR (p = 0.001, OR = 1.86, 95%CI = 1.34–2.59). Parental reported breastfeeding (six months) was positively associated with current AR (p = 0.019, OR = 1.28, 95%CI = 1.04–1.57). The frequency of truck traffic on the street of residence was positively associated with the prevalence of current AR for both the occasional truck traffic group (p = 0.007, OR = 1.39, 95%CI = 1.09–1.76) and the always truck traffic group (p = 0.001, OR = 1.92, 95%CI = 1.42–2.58), as shown in Tables 2 and 3.

In the children aged 13–14 years, parental history of atopy was not significantly related to an increased risk of current AR. Current use of paracetamol, however, was associated with increased risk of current AR (p = 0.004, OR = 1.57, 95%CI = 1.16–2.14). Only current cat exposure was associated with increased risk of current AR (p = 0.015, OR = 1.32, 95%CI = 1.05–1.64). The frequency of truck traffic on the street of residence was also positively associated with the prevalence of current AR in both the occasional truck traffic group (p = 0.032, OR = 1.25, 95%CI = 1.02–1.54) and the always truck traffic group (p < 0.001, OR = 1.62, 95%CI = 1.24–2.13), as shown in Tables 2 and 3.

Discussion

The results from our study showed the prevalence of current AR in the children aged 6–7 years to be 15.0%. When compared to ISAAC phase III in the Bangkok area at 13.4%, there was a slightly but significantly increased prevalence in the younger age group (p = 0.006). In this study, the prevalence of current AR in the 13–14-year age group was 17.5%. This decrease was significant when compared to ISAAC phase III in Bangkok (23.9%, p = 0.006). The mean global prevalence of current AR in both age groups was 9.1% and 16%, respectively, in which the Asia-Pacific prevalence was 5.8% and the ISAAC phase III prevalence was 14.5%. The results of our study so far show a higher percentage in both prevalences.

Our study confirms that parental atopy is a risk factor for the development of AR. These results are consistent with the findings of other studies. Both genetic and environmental factors play important roles in the etiology of AR. It is likely that there is a multilevel interaction between genetic and environmental factors.

This study did not find any association between antibiotic use in the first year of life and later AR. We found a positive relation between current consumption of paracetamol and the prevalence of current AR. There is a dose-related association between acetaminophen use and AR in children. The association of paracetamol with allergic disease is possible due to the depletion of glutathione. This is a result of the pharmacokinetics of this drug, leaving the respiratory mucosa with inadequate antioxidant protection. This mechanism could explain the possible association between paracetamol consumption and the prevalence of the symptoms of rhinitis in our patients.

Our results show that LRTI in the first year of life was positively associated with current AR. Respiratory infections are among the major causes of hospitalization and pediatric medical consultation, and they are directly associated with mortality in children. Allergic children showed a significantly higher number of respiratory infections in comparison with the non-allergic group. Epidemiological studies have investigated significant relationships between AR and LRTI.

In phase III of ISAAC, there was no consistent association between breastfeeding in the first year of life and rhinoconjunctivitis in 6–7-year-old children. However, breastfeeding was associated with reduced prevalence of current symptoms of severe rhinoconjunctivitis. Our results suggest that breastfeeding (six months) was associated with current AR. Several studies have shown that breastfeeding in developing countries is associated with protection against infections, particularly gastric infection and diarrhea. The immunological properties of breast milk are significant contributing factors to infant health in poor countries. Breastfeeding is therefore rightly promoted by authorities such as the World Health Organization.

ISAAC phase III showed that early-life exposure to cats is a risk factor for symptoms of rhinoconjunctivitis in 6–7-year-old children. Current exposure to cats and dogs combined, and only to dogs, is a risk factor for symptom reporting by 13–14-year-old adolescents worldwide. The Melbourne Atopy Cohort study (MASC) showed no evidence that exposure to cats and dogs at birth increases the risk of allergic disease in high-risk children. The Childhood Origins of ASThma (COAST) showed associations between allergen-specific sensitization and rhinitis. At one year, sensitization to cats was the only aeroallergen associated with an increased risk of rhinitis at 6 years of age. At age 6 years, sensitization to all allergens tested except cockroach was associated with concurrent rhinitis.

In this study, we found a positive global relationship between childhood symptoms of current AR and self-reported frequency of truck traffic on the street of residence. The associations were remarkably similar in different parts of the world in the two age groups studied and when using a self-completed questionnaire and a parent-completed questionnaire for 6–7-year-old children. A recent study from Italy found that self-reported traffic density in the area of residence was clearly associated with nitrogen dioxide, which was 39 µg/m³ when self-reported traffic was “absent,” 44 µg/m³ when “low,” 48 µg/m³ when “intermediate,” and 52 µg/m³ when “high.” First, there are now several published studies that have used objective measures of exposure and effect and found similar relationships between truck traffic exposure or other measures of exposure to vehicular traffic and respiratory and allergic symptoms in children. Second, these studies were conducted mostly in Western Europe and North America, and in ISAAC phase III the associations found in these regions were not different from those found in other parts of the world. One could argue that concern about possible adverse effects on respiratory health by traffic fumes is different in different parts of the world, so one would not expect to see a universal association if responder bias played much of a role. Third, the associations were similar for the
13–14-year-olds and the 6–7-year-olds, despite the fact that the teenagers completed the questionnaires themselves, whereas the parents completed the questionnaires for the 6–7-year-olds. We can only speculate about what factors influence the remaining heterogeneity of exposure–response relationships between participating centers. There is experimental evidence to support that diesel particles may enhance allergic sensitization to common inhalant allergens. 

The major strengths of our study included standardized written core questionnaires (GAN 2016) for AR modified from ISAAC questionnaires, a well-established and standardized protocol, and a high response rate. One limitation of our study is that it is cross-sectional, which limits our ability to determine causation. Another limitation is that symptoms of AR were self-reported in the questionnaire; therefore, we could not confirm with physical examination and laboratory investigations.

In conclusion, our study shows that the prevalence of AR remained high in both age groups. Our data confirm that a family history of atopy, LRTI in the first year of life, breastfeeding (six months), current paracetamol use, exercise, current cat exposure, and truck traffic on the street of residence are important and significant risk factors for AR symptoms. This study may serve as evidence-based health education for parents to reduce the prevalence of AR by proper management of common disease (current use of paracetamol, LRTI in the first year of life, asthma, eczema) and environmental control (pets and truck traffic on the street of residence). More detailed studies are needed on the risk factors of AR.

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References


A novel allergen-specific therapy with regulatory T cells induced by CD40-silenced dendritic cells

Motohiko Suzuki, Makoto Yokota, Shinya Ozaki, Yoshihisa Nakamura

Abstract

**Background:** We previously reported that dendritic cells (DCs) transfected with CD40 siRNA and pulsed by ovalbumin (OVA) (CD40-silenced OVA DCs) inhibited allergic responses through facilitation of regulatory T cells (Tregs). However, to our knowledge, no prior study has examined allergen-specific therapy by administration of siRNA-induced Tregs for the control of allergy.

**Objective:** We aimed to investigate the effect of Tregs induced in vitro on allergic responses and symptoms in vivo.

**Methods:** Mice were treated with Tregs (OVA DCs-induced Tregs) induced by CD40-silenced OVA DCs or Tregs (nonantigen DCs-induced Tregs) induced by DCs transfected with CD40 siRNA and pulsed with no antigen, and the effects of these Tregs on allergic responses were estimated.

**Results:** Administration of nonantigen DCs-induced Tregs prevented not only OVA-induced allergy but also keyhole limpet hemocyanin-induced allergy. Administration of OVA DCs-induced Tregs significantly reduced the number of sneezes and nasal rubbing movements, eosinophilia in the nasal mucosa, and the level of OVA-specific IgE in mice with OVA-induced allergy, compared with CD40-silenced nonantigen DC-induced Tregs in numbers 20 times greater, even in mice with established allergic rhinitis. Furthermore, Tregs induced by CD40-silenced DCs pulsed with Cry j 1, a major allergen of Japanese cedar pollen, inhibited Japanese cedar-induced allergy.

**Conclusions:** This study shows for the first time that both antigen-independent Tregs and antigen-specific Tregs can be induced by siRNA, and that therapy with siRNA-induced Tregs inhibits allergic responses and symptoms. It also shows that antigen-specific Tregs have more potent effects in inhibiting allergic responses than antigen-nonspecific Tregs.

**Key words:** Regulatory T cells, Allergy, CD40, siRNA, Dendritic cells.

From: Departments of Otorhinolaryngology, Nagoya City University

Corresponding author: Motohiko Suzuki
Departments of Otorhinolaryngology, Nagoya City University
1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya, 467-8601, Japan
E-mail: suzu-mo@med.nagoya-cu.ac.jp

Introduction

CD40 is an integral membrane protein in dendritic cells (DCs) that activates T cells. Blockade of the CD40-CD40L interaction is a potent tolerance-inducing strategy, while the inhibition of this interaction suppresses T cell responses and generates regulatory T cells (Tregs).

RNA interference using small interfering RNA (siRNA) induces specific silencing of gene expression, and is a potent, selective, and easy method. Andrew Fire and Craig Mello received the Nobel Prize in Medicine for this discovery. Silencing gene expression by siRNA is more useful and promising than conventional silencing strategies by gene or antibody, such as blocking antibody, blocking protein, antisense oligonucleotide, and ribozymes.

We previously reported that vector expressing siRNA specific for CD40 (CD40 siRNA) inhibits allergic responses not only as a means of prevention but also as treatment. However, direct administration of vector expressing siRNA may induce complications, because it is an antigen-nonspecific therapy and the vector or siRNA may change immune responses in vivo. We also showed that administration of CD40-silenced antigen-specific dendritic cells (DCs), transfected with CD40 siRNA but not vector CD40 siRNA and pulsed by antigen in vitro,
inhibited allergic responses and symptoms antigen-specifically. However, CD40-silenced antigen-specific DCs may lead to unexpected complications in vivo, since siRNA in CD40-silenced DCs may cause unexpected problems. We additionally documented that CD40-silenced DCs induce facilitation of CD4+CD25+ Tregs in vivo. Furthermore, induction of Tregs by CD40-silenced DCs is not always the same by the conditions in vivo. Considering this, direct administration of antigen-specific CD4+CD25+ Tregs, induced by siRNA in vitro, is an attractive strategy for safer and more effective control of allergic diseases. To our knowledge, however, therapy with antigen-specific CD4+CD25+ Tregs induced by siRNA in vitro has not been reported for the control of allergy, and its usefulness is not known.

The generation of Tregs with anti-CD3/CD28 antibodies in vitro has been reported. However, these are not antigen-specific Tregs. Antigen-specific Tregs are attractive for the treatment of allergy, since antigen-non-specific Tregs may affect various immune responses and contribute to a range of diseases, including cancer. It has been also reported that induced-Tregs generated by anti-CD3/CD28 antibodies differ from those induced by physiological-like activation with antigen/ APC.

In this study, we examined the effect on allergic diseases of CD4+CD25+ Tregs induced by antigen-specific DCs transfected with siRNA in vitro. The results showed that administration of ovalbumin (OVA)-specific CD4+CD25+ Tregs, induced by DCs transfected with CD40 siRNA and pulsed with OVA in vitro, inhibited allergic responses and symptoms in mice with allergic rhinitis, and that CD40-silenced DCs pulsed without antigen induced antigen-non-specific Tregs. It was also shown that antigen-specific Tregs were more potent in inhibiting allergic responses and symptoms than antigen-non-specific Tregs.

Methods

Generation of bone marrow-derived DCs and gene silencing by siRNA

DCs were generated from bone marrow progenitor cells, as previously described. These DCs were transfected with transfection reagent alone (No siRNA DCs), siRNA (Control siRNA) specific to the Luciferase gene GL2 Duplex siRNA (Control DCs), or siRNA (CD40 siRNA, UUCUCAGCCCAUGGAAACA) specific to CD40. DCs transfected with CD40 siRNA were pulsed with OVA (CD40-silenced OVA DCs) or without OVA (CD40-silenced nonantigen DCs), as described previously. DCs transfected with CD40 siRNA were also pulsed with Cry j 1, a major allergen of Japanese cedar (Cryptomeria japonica) pollen, (CD40-silenced Cry j 1 DCs) by the same method. Cry j 1 was purified by the method previously reported.

Generation of Tregs in vitro

Mouse naïve CD4+ T cells were isolated from splenic cells of six to eight week-old male BALB/c mice using a Mouse Naïve CD4+ T Cell Isolation Kit (R&D Systems, CA). Mouse naïve CD4+ T cells (3 × 10^6/mL) were co-cultured with 6 × 10^6/mL No siRNA DCs, Control DCs, CD40-silenced nonantigen DCs, CD40-silenced OVA DCs, or CD40-silenced Cry j 1 DCs for 5 days in 2 mL of complete medium, RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 50 µM 2-ME, and 10% FCS supplemented with TGF-β (5 ng/mL) and IL-2 (50 IU/mL). CD4+CD25+ T cells were collected using a MACS negative CD4 isolation kit and anti-CD25 MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany).

Immunization and Treatment

Six to eight week-old male BALB/c mice (Japan SLC Inc., Shizuoka, Japan) were injected intravenously with PBS alone, Tregs (4 × 10^6 or 4 × 10^6 cells/mouse) induced by CD40-silenced nonantigen DCs, or Tregs (4 × 10^6 cells/mouse) induced by CD40-silenced OVA DCs on day 1. Mice were also injected intraperitoneally (i.p.) with 4 mg Al(OH)_3 and 10 µg ovalbumin (OVA) twice on days 2 and 15. Each group consisted of five mice. The same mice were challenged intranasally (i.n.) on days 21 through 27 with OVA (100 µg). Samples were collected on day 28.

In the second experiment, the protocol was the same as in the above experiment except that mice received PBS alone, Tregs (4 × 10^6 or 4 × 10^6 cells/mouse) induced by CD40-silenced nonantigen DCs, or Tregs (4 × 10^6 or 4 × 10^6 cells/mouse) induced by CD40-silenced OVA DCs and that mice were injected i.p. with 4 mg Al(OH)_3 and keyhole limpet hemocyanin (KLH), but not OVA, on days 2 and 15 and challenged i.n. on days 21 through 27 with KLH.

In the third experiment, mice were sensitized with OVA (10 µg) and 2 mg Al(OH)_3 intraperitoneally on days 1 and 14, and then the same mice were challenged intranasally with OVA (100 µg) on days 18 through 24. Intravenous administration of PBS alone, Tregs induced by CD40-silenced nonantigen DCs (4 × 10^6 or 8 × 10^6 cells/mouse), or Tregs by CD40-silenced OVA DCs (4 × 10^6 cells/mouse), was performed on day 26. These mice were then re-challenged intranasally on days 27 through 32 with OVA (100 µg).

In the fourth experiment, mice were sensitized with Cry j 1 (3 µg) and 2 mg Al(OH)_3 intraperitoneally on days 1 and 14, and then the same mice were challenged intranasally with Cry j 1 (2 µg) on days 18 through 24. Intravenous administration of PBS alone, Tregs induced by CD40-silenced nonantigen DCs (8 × 10^6 cells/mouse), or Tregs by CD40-silenced Cry j 1 DCs (4 × 10^6 cells/mouse), was performed on day 26. These mice were then re-challenged intranasally on days 27 through 32 with Cry j 1 (3 µg).

This study was approved by Research Ethics Committee in Nagoya City University. Mice were housed in an environmentally-controlled animal facility at Nagoya City University in Japan. The protocols were in accordance with the Guidelines for Care and Use of Animals of Nagoya City University. Every effort was made to minimize the discomfort of the animals.

Cry j 1- specific T cell response

CD4+CD25 T cells and CD11c cells were isolated from spleen using MACS beads (Miltenyi Biotec). Spleen CD4+CD25 T cell (2 × 10^6 cells/mL) and DC (2 × 10^6 cells/mL) suspensions were cultured for 72 h and stimulated with 10 µg/mL Cry j 1 antigen.
OVA-specific T cell response
Spleenic cells isolated by gradient centrifugation over Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) were cultured in 96-well plates at a concentration of 4 x 10^5 cells/well for 72 h in the presence of 100 μg/mL OVA antigen.

Measurement of IL-2 production
Spleen CD4^+CD25^+ T cell (2 x 10^5 cells/mL) and DC (2 x 10^5 cells/mL) transsected with or without CD40 siRNA suspensions were cultured for 72 hours, stimulated with 10 μg/mL Cry j 1. Quantities of IL-2 cytokines in the culture supernatants were determined by using a sandwich ELISA. Plates were coated with anti-mouse IL-2 (BioLegend, San Diego, CA). The culture supernatant was then added, and the plates were incubated with the second antibody of biotinylated anti-mouse IL-2 (BioLegend). Standard curves were generated by using recombinant cytokines.

Measurement of OVA-specific, KLH-specific, and Cry j 1-specific IgE in sera
Titers of specific IgE were measured by ELISA. Briefly, ELISA plates were coated with anti-mouse IgE monoclonal antibody (Yamasa, Tokyo, Japan). Non-specific binding was blocked and sera were added. After washing with wash buffer, biotinylated OVA, KLH, or Cry j 1 was added to the well. The plates were then incubated with avidin-peroxidase at 37°C for an hour after washing. The TMB microwell peroxidase substrate system (KPL, Gaithersburg, MD) was used, and optical density (O.D.) was measured at 450 nm.

Nasal allergic symptoms
Immediately after the last nasal challenge, the number of sneezes and nasal rubbing movements was counted for 20 min according to the method previously reported.11

Pathology
The heads were decalcified and sectioned. Three micrometer thick sections of nasal tissue were stained with Luna staining. The number of eosinophils in the nasal mucosa of the nasal septum was counted microscopically in a field of view at 400× magnification. The observer was blinded to treatment when counting the number of eosinophils.

Statistical analysis
Data are expressed as means ± SEM. Statistical comparisons between groups were performed using one-way ANOVA followed by the Newman-Keuls Test. Differences with P-values less than 0.05 were considered significant.

Results
Prevention of OVA-induced allergy with CD40-silenced DC-induced OVA Tregs
We investigated whether Tregs induced by CD40-silenced OVA DCs in vitro could prevent OVA-induced allergy. Mice that received PBS, CD40-silenced nonantigen DC-induced CD4^+CD25^+ cells, or CD40-silenced OVA DC-induced CD4^+CD25^+ cells were sensitized and challenged with OVA as described in Methods (treatment on day 1, sensitization on days 2 & 15, challenge on days 21-27, sample collection on day 28). The number of sneezes and nasal rubbing movements was counted immediately after the last nasal challenge to examine the effect of these T cells on nasal allergic symptoms. CD40-silenced OVA DC-induced Tregs significantly decreased the number of sneezes and nasal rubbing movements compared with the other groups (Figure 1A and B). Although CD40-silenced nonantigen DC-induced T cells at a concentration of 4 x 10^6 cells/mouse did not reduce these symptoms, CD40-silenced nonantigen DC-induced T cells at levels 10 times greater and more (4 x 10^7 cells/mouse and 8 x 10^6 cells/mouse) significantly inhibited these symptoms. However, there were no significant differences in symptom inhibition between CD40-silenced nonantigen DC-induced Tregs at levels of 4 x 10^6 cells/mouse and 8 x 10^6 cells/mouse.

Next, the number of eosinophils in the nasal septum was counted to evaluate eosinophilia, which is associated with allergic symptoms and allergic responses in the nose. The number of eosinophils infiltrating the nasal mucosa in mice inducted with Tregs induced by CD40-silenced OVA DCs was

Figure 1. Prevention effects of allergy by CD4^+CD25^+ cells induced by CD40-silenced OVA DCs.
Five mice were injected intraperitoneally and challenged intranasally with OVA after treatment of PBS alone, CD40-silenced nonantigen DC-induced CD4^+CD25^+ cells (CD40 Non, 4 x 10^5 “X1”, 4 x 10^6 “X10”, or 8 x 10^6 “X20”, cells/mouse), or CD40-silenced OVA DC-induced CD4^+CD25^+ cells (CD40 OVA, 4 x 10^5 cells/mouse). The number of sneezes (A) and nasal rubbing movements (B) was counted after the last nasal challenge.
The number of eosinophils

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<tr>
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<td>10</td>
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Figure 1. (Continued)

(C) Eosinophilia of the nasal septum. (D) The level of OVA-specific IgE in sera. The level of IL-4 (E) and IL-5 (F) production from splenic splenocytes stimulated by OVA was measured by ELISA. ** P < 0.01 versus groups of PBS alone and CD40 Non X1. ##P < 0.01 versus groups of CD40 Non (X10, X20). Experiments were repeated 3 times with similar result.

significantly fewer than that in mice with PBS alone or Tregs induced by CD40-silenced nonantigen DCs (Figure 1C). CD40-silenced nonantigen DC-induced Tregs at levels of $4 \times 10^6$ cells/mouse or $8 \times 10^6$ cells/mouse also significantly inhibited this eosinophilia, whereas CD40-silenced nonantigen DC-induced Tregs at the level of $4 \times 10^5$ cells/mouse did not (Figure 1C).

We also measured OVA-specific IgE in sera by ELISA, since IgE is associated with allergic reactions. CD40-silenced nonantigen DC-induced Tregs at levels of $4 \times 10^5$ or $8 \times 10^5$ cells/mouse also significantly suppressed the level of OVA-specific IgE, although CD40-silenced nonantigen DC-induced Tregs at the level of $4 \times 10^5$ cells/mouse cells/mouse did not. Tregs produced by CD40-silenced OVA DCs inhibited OVA-specific IgE significantly more than the other groups (Figure 1D). These data suggest that Tregs induced by CD40-silenced OVA DCs prevent production of OVA-specific IgE.

IL-4 and IL-5 play important roles in the development of allergic diseases. In order to investigate the effect of Tregs induced by CD40-silenced OVA DCs on cytokine production, we measured the production of IL-4 and IL-5 from splenic T cells stimulated with OVA in vitro. There were no significant differences between mice received PBS alone and CD40-silenced nonantigen DC-induced Tregs at levels of $4 \times 10^5$ cells/mouse in the productions of IL-4 and IL-5. The levels of IL-4 and IL-5 produced in mice that received Tregs induced by CD40-silenced OVA DCs were significantly lower than those in mice that received PBS or Tregs induced by CD40-silenced nonantigen DCs (Figure 1E and F). This suggests that OVA-specific Tregs suppress the production of Th2 cytokines, which may contribute to the prevention of allergy.

**No preventive effect of Tregs induced by CD40-silenced OVA DCs on KLH-induced allergy**

To investigate antigen specificity, we examined whether Tregs induced by CD40-silenced OVA DCs in vitro can inhibit allergic responses and symptoms caused by KLH. Mice received PBS, CD40-silenced nonantigen DC-induced Tregs, or CD40-silenced OVA DC-induced Tregs were sensitized and challenged with KLH as described in Methods (treatment on day 1, sensitization on days 2 & 15, challenge on days 21-27, sample collection on day 28). Administration of Tregs induced by CD40-silenced OVA DCs did not significantly inhibit the number of nasal sneezes, nasal rubbing movements, or eosinophils at the nasal septum and the level of KLH-specific IgE compared with mice that received PBS alone (Figure 2A-D). These findings suggest that Tregs induced by CD40-silenced OVA DCs inhibit allergen reactions and symptoms in an antigen-specific manner.

Administration of CD40-silenced nonantigen DC-induced Tregs ($4 \times 10^5$ cells/mouse) inhibited the number of nasal sneezes, nasal rubbing movements, and eosinophils at the nasal mucosa and KLH-specific IgE levels in sera compared with the other groups (Figure 2A-D). These results suggest
Figure 3. Therapeutic effects of CD4+CD25+ Tregs induced by CD40-silenced OVA DCs in vitro on established allergic rhinitis.

Five mice with OVA-induced allergic rhinitis were treated with PBS alone, CD40-silenced nonantigen DC-induced CD4+CD25+ cells (CD40 Non, 4 × 10^6 “X1” or 8 × 10^6 “X20” cells/mouse), or CD40-silenced OVA DC-induced CD4+CD25+ cells (CD40 OVA, 4 × 10^6 “X1” or 4 × 10^6 “X10” cells/mouse). The number of sneezes (A) and nasal rubbing movements (B) was counted after the last nasal challenge.

Figure 2. No allergy prevention effect from CD4+CD25+ Tregs induced by CD40-silenced OVA DCs.

Five mice were injected intraperitoneally and challenged intranasally with KLH after treatment with PBS alone, CD40-silenced nonantigen DC-induced CD4+CD25+ cells (CD40 Non, 4 × 10^5 “X1” or 4 × 10^6 “X10” cells/mouse), or CD40-silenced OVA DC-induced CD4+CD25+ cells (CD40 OVA, 4 × 10^5 “X1” or 4 × 10^6 “X10” cells/mouse). The numbers of sneezes (A) and nasal rubbing movements (B) were counted after the last nasal challenge. (C) Eosinophilia of the nasal septum. (D) The level of KLH-specific IgE in sera. ** P < 0.01 versus groups of PBS alone, CD40 Non X1, and CD40 OVA (X1, X10). Experiments were repeated 3 times with similar result.
that CD40-silenced nonantigen DC-induced Tregs are not antigen-specific.

**Therapeutic effects of Tregs induced by CD40-silenced OVA DCs on mice with established OVA-induced allergic rhinitis**

Mice with established allergic rhinitis were treated with PBS alone, CD40-silenced nonantigen DC-induced Tregs, or CD40-silenced OVA DC-induced Tregs. After treatment, nasal re-challenge with OVA was performed (sensitization on days 1 & 14, nasal challenge on days 18-24, treatment with Tregs on day 26, nasal re-challenge on days 27-32, sample collection on day 33). The number of sneezes and nasal rubbing movements on day 24 was significantly higher than on day 17 (data not shown). Eosinophils in the nasal septum were seen on day 24, although no eosinophilia was found on day 17 (data not shown). These results suggest that mice were suffering from allergic rhinitis on day 24. There were no significant effects on the number of sneezes, nasal rubbing movements, or eosinophils in the nasal mucosa, or the level of OVA-specific IgE in sera, even when CD40-silenced nonantigen DC-induced Tregs (8 × 10^6 cells/mouse) were injected (Figure 3A-D).

Tregs induced by CD40-silenced OVA DCs in vitro significantly reduced the number of sneezes, nasal rubbing movements, and eosinophils in the nasal mucosa, and the level of OVA-specific IgE in sera, compared with the other groups, PBS alone, and Tregs induced by CD40-silenced nonantigen DCs (Figure 3A-D). These findings suggest that Tregs induced by CD40-silenced OVA DCs are therapeutically useful even for mice with established allergic rhinitis.

**Immune regulatory properties of Tregs induced by DCs (CD40-silenced Cry j 1 DCs) transfected with CD40 siRNA and pulsed with Cry j 1**

Next, we investigated Tregs induced by CD40-silenced DCs (CD40-silenced Cry j 1 DCs) pulsed with Cry j 1 but not OVA, because OVA is a food allergen but not aeroallergen. Cry j 1 is one of the major allergens of Japanese cedar pollen which cause severe allergic diseases in Japan.15-19 Bone marrow-derived DCs were transfected with CD40 siRNA or Control siRNA (Control DCs). DCs transfected with CD40 siRNA were pulsed (8 × 10^6 cells/mouse) with Cry j 1.

Figure 3. (Continued)
(C) Eosinophilia of the nasal septum. (D) The level of OVA-specific IgE in sera. ** P < 0.01 versus group of PBS alone, CD40 Non X10, and CD40 Non X20. Experiments were repeated 3 times with similar result.

Figure 4. Modulation by CD40 siRNA in vitro. (A) DCs were transfected with Control siRNA (Control DCs) or CD40 siRNA. DCs transfected with CD40 siRNA were pulsed without Cry j 1 (CD40 Non DCs) or with Cry j 1 (CD40 Cry j 1 DCs). The numbers of CD4^+CD25^+ cells induced from 3 × 10^5 naïve CD4^+ cells by Control DCs, CD40 Cry j 1 DCs, and CD40 Non DCs were examined. (B) The percentage of CD25^+Foxp3^+ T cells in CD4^+ T cells after co-culture of T cells and DCs. (C) Quantity of IL-2 production after co-culture of T cells and DCs. ** P < 0.01 versus group of Control DCs. Experiments were repeated 3 times with similar result.
with Cry j 1 (CD40-silenced Cry j 1 DCs) or no antigen (CD40-silenced nonantigen DCs). Naïve T cells, separated from splenic T cells in naïve mice as described in Methods, were co-cultured with Control DCs, CD40-silenced nonantigen DCs, or CD40-silenced Cry j 1 DCs. Although we assessed the number of CD4+CD25+ cells were induced from 3 × 10^6 naïve CD4+ cells, the number of CD4+CD25+ cells induced by CD40-silenced Cry j 1 DCs or CD40-silenced nonantigen DCs were significantly higher than that by Control DCs. (Figure 4A). The percentage of CD25+Foxp3+ cells in CD4+ T cells induced by CD40-silenced nonantigen DCs and CD40-silenced Cry j 1 DCs were significantly higher compared with those induced by Control DCs (Figure 4B). And we investigated whether CD4+CD25+ cells induced by CD40-silenced Cry j 1 DCs could affect IL-2 production in order to examine the mechanism of Treg induction, since the association between IL-2 production and Treg expansion has been reported.20,21 Cry j 1-specific T cell response was generated by a co-culture of DCs and CD4+CD25+ T cells isolated from the spleen in mice sensitized with Cry j 1 antigen. Quantity of IL-2 in the supernatant was measured by ELISA. Consequently, IL-2 production was significantly inhibited by CD40-silenced nonantigen DCs or CD40-silenced Cry j 1 DCs (Figure 4C).

**Therapeutic effects of Tregs induced by CD40-silenced Cry j 1 DCs on mice with established Cry j 1-induced allergic rhinitis**

We assessed the effects of siRNA-induced Tregs on allergic diseases caused by aeroallergen, Japanese cedar pollen. Mice with allergic rhinitis were treated with PBS alone, CD40-silenced nonantigen DC-induced Tregs, or CD40-silenced Cry j 1 DC-induced Tregs. After treatment, nasal re-challenge with Cry j 1 was performed (sensitization on days 1 & 14, nasal challenge on days 18-24, treatment with Tregs on day 26, nasal re-challenge on days 27-32, sample collection on day 33). No eosinophilia in the nasal septum was found on day 17, whereas eosinophilia was seen on day 24 (data not shown). The numbers of sneezes and nasal rubbing movements on day 24 were significantly higher than those on day 17 (data not shown). These suggest that allergic rhinitis was established on day 24. After treatment with CD40-silenced nonantigen DC-induced Tregs, there were no significant effects on the number of sneezes, nasal rubbing movements, eosinophilia in the nasal mucosa, and

![Figure 5. Therapeutic effects of CD4+CD25+ Tregs induced by CD40-silenced Cry j 1 DCs in vitro on established allergic rhinitis.](image-url)

Five mice with Cry j 1-induced allergic rhinitis were treated with PBS alone, CD40-silenced nonantigen DC-induced CD4+CD25+ cells (8 × 10^6 cells/mouse, CD40 Non Tregs) or CD40-silenced Cry j 1 DC-induced CD4+CD25+ cells (4 × 10^6 cells/mouse, CD40-Cry j 1 Tregs). The number of sneezes (A) and nasal rubbing movements (B) was counted after the last nasal challenge. (C) Eosinophilia of the nasal septum. (D) The level of Cry j 1-specific IgE in sera. ** P < 0.01 versus group of PBS alone, ## P < 0.01 versus group of CD40 Non Tregs. Experiments were repeated 3 times with similar result.
the level of Cry j 1-specific IgE in sera (Figure 5A-D). However, Tregs induced by CD40-silenced Cry j 1 DCs in vitro significantly reduced the number of sneezes, nasal rubbing movements, and eosinophilia in the nasal mucosa, and the level of Cry j 1-specific IgE in sera, compared with other groups, PBS alone, and Tregs induced by CD40-silenced nonantigen DCs (Figure 5A-D). These findings suggest that Tregs induced by CD40-silenced Cry j 1 DCs are therapeutically useful for mice with allergic rhinitis caused by Japanese cedar pollen.

Discussion

Administration of Tregs induced by CD40-silenced nonantigen DCs before sensitization significantly reduced allergic responses and symptoms not only in OVA-induced allergy but also in KLH-induced allergy. These results suggest that Tregs induced by CD40-silenced nonantigen DCs are antigen-non-specific Tregs. Patients who suffer from sensitization to multiple allergens are increasing.23 Antigen-specific therapy for these patients is not easy, nor is it applicable for patients with an unknown causative allergen. Thus, CD40 silenced nonantigen DC-induced Tregs may be an alternative, antigen-independent therapy for the prevention of allergic diseases.

Although blockade of CD40-CD40L interaction induce Tregs,24,25 the underlying mechanism of Treg expansion by blockade of CD40-CD40L is not known.24 However, low-dose IL-2 expands CD4+ regulatory T cells with a suppressive function in vitro.26 Both blockade of B7-CD28 and CD40-CD40L also activated Foxp3+ regulatory T cells and reduced IL-2 production.27 When CD25+ CD4+ T cells compete with other cells for IL-2, CD4+CD25+ T cells further up-regulate the CD25 (IL-2R alpha chain).28 And Vogel et al.29 assumed that the low amount of IL-2 is enough for the survival of CD4+Foxp3+ cells, but not enough for the survival of CD4+Foxp3- cells. This study showed that blockade of only CD40-CD40L pathway inhibited IL-2 productions. These result suggest that blockade of CD40-CD40L induces expansion of CD4+Foxp3+ Tregs through reduction of IL-2 production.

We previously reported that CD40-silenced OVA DCs inhibited allergic reactions and symptoms. However, CD40-silenced OVA DCs may induce unexpected problems in vivo. CD40 siRNA may go out of DCs and induce problems such as inhibition of CD40 gene on other cells, interferon response, and off-target effect, although these have been not reported. If deficiency of CD40-CD40L interaction occurs in vivo, this may lead susceptibility to infection,26,27 like hyper IgM syndrome,28 dsRNA, less than 30 bp in length, are generally believed to avoid interferon responses.29 However, interferon response should be paid attention to even in siRNA, since siRNA could interferon response30,31 and since the threshold of dsRNA length to induce interferon responses varies by cell types.29 In future, various 'Treg phenotype may be revealed. Even if siRNA-induced Tregs include various Treg phenotype, it may be possible to collect only specific phenotype before administration in time to come. The advantages of this novel therapy with siRNA-induced Tregs presented herein include: 1) no interferon responses caused by siRNA; 2) no off-target effects by siRNA; 3) no inhibition of CD40 gene expression in vivo by CD40 siRNA; 4) no unexpected problems by siRNA or siRNA-transfected DCs; 5) higher stability in the numbers of siRNA-induced Tregs administered (induction of Tregs by CD40-silenced DCs is not always the same by the conditions in vivo), and 6) possibility to select specific Treg phenotype before administration, compared with therapy with siRNA-transfected DCs.

In this study, we report a novel antigen-specific therapy for the control of allergic diseases, using Tregs induced by CD40-silenced antigen-specific DCs transfected with CD40 siRNA in vitro, and siRNA-induced antigen-nonspecific Tregs for the prevention of allergic diseases. Furthermore, antigen-specific Tregs induced by siRNA-modulated DCs are attractive since they have more potent inhibiting effects on allergic responses and symptoms than antigen non-specific Tregs.

Financial disclosure

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Conflict of interest

None

Authors’ contributions

Motohiko Suzuki and Yoshihisa Nakamura designed the study. Motohiko Suzuki and Makoto Yokota wrote the manuscript. Makoto Yokota and Shin'ya Ozaki contributed to data collection. Shin'ya Ozaki and Yoshihisa Nakamura performed the statistical analysis and interpretation of the results. All authors read and approved the final manuscript.

References

Instructions for authors

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