Use of polymerase chain reaction for detection of adenovirus in children with or without wheezing

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Eighty percent of asthma attacks in children are accompanied by an upper respiratory tract viral infection. Adenovirus is one of the major viral causes of childhood bronchiolitis.

As the polymerase chain reaction (PCR) is the most sensitive technique for documenting viral respiratory infections, the PCR method was performed on the throat swab samples of asthmatic children with and without wheezing to investigate the presence of the adenovirus genome in the upper respiratory tract.

The frequencies of adenovirus in asymptomatic and symptomatic asthmatic patients, healthy controls and wheezy children were as follows: 33.3%, 71.4%, 37% and 62.96%, respectively. The adenovirus was detected in a significantly higher percentage in the upper airways of patients with asthma exacerbation and in children with wheezing than in patients without asthma exacerbation and in the healthy controls (p<0.05). The frequency of adenovirus was not different between asthmatic patients receiving or not receiving inhaled corticosteroid.

Adenovirus has the potential to precipitate asthma exacerbations in asthmatic patients; its frequency was not affected by the treatment of inhaled corticosteroid.

Key words: adenovirus, polymerase chain reaction, wheezing, asthma, upper respiratory tract.

Viral infections have long been implicated in triggering asthmatic attacks among both children and adults1. RNA viruses such as rhinoviruses are thought to be the most common agents2. A double-stranded DNA virus, adenovirus, is one of the major viral causes of childhood bronchiolitis3. However, there is little if any intervention regarding the role of adenoviruses in asthmatic exacerbations. In order to investigate whether adenoviruses may be associated with exacerbation of wheezing in childhood, we conducted a study using polymerase chain reaction (PCR) technology, which is believed to be a more sensitive screening method than traditional diagnostic methods4.

Material and Methods

Nasopharyngeal swabs were obtained from asthmatic patients, healthy controls, and recurrent wheezy children, in total, four different groups. Group I: Asymptomatic asthmatic children, Group II: Symptomatic asthmatic children, Group III: Healthy children, Group IV: Non-asthmatic recurrent wheezy symptomatic children.

The PCR method was performed on all throat samples in order to investigate adenovirus genome in the upper respiratory tract.

Subjects

This study was conducted at Hacettepe University Faculty of Medicine between February and December 1999. The study was carried out on asthmatic children aged 6-19 years admitted to the Pediatric Allergy and Asthma Unit. All the asthmatic children (Groups I and II) had been diagnosed previously as asthmatic according to the American Thoracic Society Criteria5, and they were all being
regularly followed in the Pediatric Asthma Clinic of the University. Patients from Group I were asymptomatic during sample collection and had experienced no respiratory infection symptoms during the previous two weeks. Group II patients had wheezing at the time of sample collection which was audible without chest auscultation. Recent upper respiratory tract infections, the severity of respiratory distress, socio-economic status, active or passive smoking, and severity of asthma were not considered for inclusion criteria.

Patients of Group III, i.e. healthy controls, were selected from the Emergency Unit of our hospital. They were mainly selected from the patients admitted for trauma and were excluded if they had any recent respiratory infection during the previous two weeks. They were questioned using the International Study of Asthma and Allergies in Childhood (ISAAC) questionnaire in order to ensure they had no recurrent wheezing and/or asthma history.

Patients of Group IV were recruited from the Emergency Unit, to which they admitted with wheezing and respiratory distress. In order to recruit post-viral recurrent wheezy children, it was required to have a recurrent wheezing history, i.e. at least two wheezing episodes prior to admittance, and to have no history of asthma diagnosis by their local physician.

Five (18.5%) of the asymptomatic asthmatics and 16 (57.1%) of the symptomatic asthmatics had been on regular low-to-moderate dose inhaled corticosteroid (ICS) treatment (fluticasone 100-500 mcg, budesonide 100-800 mcg) for at least six months. None of Group IV was on ICS, but some were using short-acting beta-2 agonists (n=4, 14%) and/or cromolyn sodium (n=6, 22%).

**Sample Collection**

Throat swab specimens were collected with Dacron polyester-tipped swabs with plastic shifts. After collection, swabs were placed into a screw-capped tube containing 5.0 ml of TE (10 mM Tris-HCl, 1.0 mM EDTA, pH 8.0) buffer. All specimens were kept at 4°C until processing.

**DNA Extraction**

For DNA extraction the tubes were vortexed and swabs were removed. Supernatants were discarded after centrifugation. The final pellets were resuspended in 1 ml TE buffer. The suspensions were taken into 1.5 ml microcentrifuge tubes as 500 µl suspension to each tube. Specimens were washed twice in TE buffer and the pellet resuspended with 100 µl TE buffer. Tubes were then subjected to a 20 min incubation in a heat block at 95°C and one freeze/thaw cycle at -70°C. Tubes were kept at -20°C until PCR.

**PCR Method**

For the detection of adenovirus DNA by PCR, primers that amplify 134-bp fragment of the hexon gene were used. Primer 1 (5’-GCC GAG AAG GGC GTG CGC AGG TA-3’) and primer 2 (5’-ATG ACT TTT GAG GTG GAT CCC ATG GA-3’) are specific for all adenoviruses and cannot differentiate the groups or serotypes. Amplifications were performed in a final volume of 50 µl (5 µl specimen in 45 µl reaction mixture) in 0.2 ml reaction tubes. The reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3.0 mM MgCl, 200 µmol of each dATP, dGTP, dCTP and dTTP, 0.5 µmol of each primer and 2.5 U of Taq polymerase. All tubes were placed in a thermocycler (MJ Research, PTC 200) programmed as follows: initial incubation for 5 min at 94°C, 45 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 90 sec. An additional incubation at 72°C for 5 min was added for the final extension after the final cycle. Amplified products were examined after 1.5% agarose gel electrophoresis at 100 V and staining with ethidium bromide (Fig. 1).

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Fig. 1. Agarose gel electrophoresis of adenovirus polymerase chain reaction (PCR) products amplified from throat specimens.

M: 100 bp ladder.
Lanes 1, 3, 4, 5: Adenovirus genome-positive patients.
Lanes 2, 6: Adenovirus-negative patients.
Processing of specimens, reaction mixture preparation, amplification and detection steps were conducted in separate rooms, and all processing was conducted in separate rooms under DNA-free cabinets with laminar air flow to avoid contamination. Positive and negative reaction controls as well as the spiked inhibition controls were also performed. Positive specimens were re-extracted and amplified by PCR in order to ensure positive results.

**Statistical Analysis**

Results were expressed as the mean ± standard error of the mean (SEM) and percentages, and groups were compared using Student’s t test or Pearson $\chi^2$ test and vassar confidence interval for means and ratios, respectively. Two-sided p values <0.05 were considered significant. All analyses were performed using the SPSS 10.0 program.

**Table I.** Major Characteristics of the Patients in the Four Different Groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Asymptomatic</th>
<th>Symptomatic</th>
<th>Healthy controls</th>
<th>Wheezy children</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>27</td>
<td>28</td>
<td>27</td>
<td>27</td>
</tr>
<tr>
<td>Mean age±SEM</td>
<td>9.80±0.54</td>
<td>9.16±0.57</td>
<td>10.62±0.4</td>
<td>3.41±0.24</td>
</tr>
<tr>
<td>Girls/Boys</td>
<td>12/15</td>
<td>12/16</td>
<td>12/15</td>
<td>10/17</td>
</tr>
<tr>
<td>ICS Treatment</td>
<td>(18.51%)</td>
<td>(57.14%)</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

ICS: Inhaled corticosteroid.

**Results**

There were no statistical differences between the four groups with respect to sex distribution. The mean ages were not different between the first three groups, whereas patients of Group IV were younger than in the other three groups. The characteristics of patients in the four different groups are shown in Table I.

Adenovirus genome was positive in 9 [33.3% (95%CI:18.6-52.1)], 20 [71.4% (52.9-84.7)], 9 [37% (21.5-55.7)] and 17 [62.9% (44.2-78.4)] of patients in Groups I, II, III and IV, respectively. The frequency of adenovirus presence among symptomatic asthmatic and wheezy children was significantly higher than in the asymptomatic asthmatics and healthy controls (p<0.05). There was no significant difference between symptomatic asthmatic and wheezy children, and between asymptomatic asthmatic and healthy controls (Table II).

**Table II.** Frequency of Adenovirus According to the Four Different Groups and to Treatment or not with Inhaled Corticosteroid

<table>
<thead>
<tr>
<th>Asymptomatic</th>
<th>Symptomatic</th>
<th>Healthy Controls</th>
<th>Wheezy Children</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>I</td>
<td>II</td>
<td>III</td>
</tr>
<tr>
<td>Number</td>
<td>27</td>
<td>28</td>
<td>27</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>9</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>(33.3%)</td>
<td>(71.4%)</td>
<td>(37%)</td>
<td>(62.9%)</td>
</tr>
<tr>
<td>95% Confidence Interval</td>
<td>18.6-52.1</td>
<td>52.9-84.7</td>
<td>21.5-55.7</td>
</tr>
<tr>
<td>Adenovirus/ICS</td>
<td>1/5</td>
<td>11/16</td>
<td>—</td>
</tr>
<tr>
<td>(20%)</td>
<td>(68%)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Adenovirus/Non-ICS</td>
<td>8/22</td>
<td>9/12</td>
<td>—</td>
</tr>
<tr>
<td>(36%)</td>
<td>(75%)</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

ICS: Inhaled corticosteroid.
In order to investigate the effect of inhaled corticosteroid on adenovirus presence, Groups I and II were each divided into two groups according to ICS use, and subgroup comparisons were conducted. In Group I, adenovirus genome was positive in one patient (20%) on ICS and in eight patients (36%) not on ICS, and the difference was not statistically significant. Similarly, in Group II, adenovirus genome was positive in 11 patients (68%) who were on ICS and in nine patients (75%) who were not, and the difference between these two subgroups again was not statistically significant. The subgroups of patients receiving or not receiving ICS and their adenovirus results are shown in Table II.

Discussion

The search for the presence of adenovirus genome in the upper respiratory tract of asthmatic children resulted in the discovery of a significant difference between the asymptomatic and symptomatic children. The frequency of adenovirus in symptomatic asthmatic patients and recurrent wheezy children is significantly higher than in asymptomatic asthmatic patients and in healthy controls. Edwards et al. demonstrated that adenovirus is responsible for a high attack rate and causes significant respiratory disease in children under seven years of age. As a result of our study, we suggest that adenovirus, a known responsible agent of respiratory symptoms, is able to precipitate wheezing in childhood and is responsible for some of the asthma attacks in childhood and adolescence. It probably has an effect on the increase of asthma severity by causing a chronic, latent inflammation in the lower airways, as recently identified by Marin.

Adenoviruses are ubiquitous and are most frequently isolated in the upper respiratory tract and stool, and there is both chronic respiratory and fecal shedding of the virus. Adenoviruses 1, 2, 5 and 6 appear to be endemic in many parts of the world. Adenovirus infection rates are far less than those of rhinovirus and respiratory syncytial virus (RSV). Furthermore, a recent study showed that among asthmatic children with exacerbations of asthma, only 4.5% of the patients had adenovirus infection as detected using PCR. Although the prevalence/incidence of adenovirus infection may be higher in our population as compared to the French population evaluated in the latter study, the rates observed in the present population may not be representative of the general population of asthmatic children. It can be suggested that adenovirus is an etiology for at least 30% of virus-induced wheezing after the subtraction of its incidence seen in cases without wheezing.

Another possible reason for the observed high incidence of adenovirus in the upper respiratory tract in our study population was reported in Freymuth et al.'s study. They reported that low-level asymptomatic respiratory shedding of adenovirus DNA is presumably more frequent in nasal samples than for the other respiratory viruses. There could be some false-positive results, and this point deserves careful consideration while repeating DNA extraction and PCR analysis. There are several possible reasons for the high incidence of adenovirus in the throat samples of our patients. Although PCR would detect more infected subjects compared to traditional diagnostic methods, the numbers obtained seems to be extremely high. First, this is probably due to selection bias, whereby a highly selective group of individuals have been studied. Second, some adenovirus serotypes are known to be shed for prolonged periods of time in the absence of symptoms. Third, the primers that we used in PCR amplification were specific for all adenoviruses. These factors may explain the high incidence of adenovirus, including in healthy children.

Gern and Corne defined that all respiratory viruses (with the exception of Coronavirus) are able to infect the lower respiratory tract as well. Recent studies have implied that viral infections of the upper respiratory tract may extend to involve the lower respiratory tract, which might be one of the mechanisms for virus-induced exacerbations of asthma. As mentioned by Johnston et al., viral respiratory infections have been associated with the most acute exacerbations of wheezing in childhood. The PCR method is associated with significantly higher rates of respiratory virus detection than either culture or immunofluorescence methods. It also identifies that there are no significant differences in the rates of viral detection between induced sputum and nasopharyngeal aspirate during acute exacerbations of asthma. All these arguments prove that PCR method using throat swab samples represents the lower airways conditions. This study supports that
adenovirus is a member of the respiratory viruses which precipitate wheezing from early infancy to adolescence.

Hogg and Yamada\textsuperscript{17,18} demonstrated that latent adenoviral infection has the potential to modify the steroid response in allergic lung inflammation in animal models by increasing the eosinophilic component of this response. Chronic or latent adenovirus infection could alter activation of transcription factors and increase expression of inflammatory mediators in the airway as well as directly inhibit the action of corticosteroids\textsuperscript{18}. The findings of the report presented by Macek et al.\textsuperscript{19} also demonstrated a high frequency of adenovirus in bronchoalveolar lavage of chronic asthmatic children who were poorly responsive to corticosteroid therapy. The results obtained from this study demonstrated that there was no tendency to high frequency of the adenovirus genome in the upper respiratory tract of the asthmatic patients receiving ICS. However, the absence of a relationship between corticosteroid usage and adenoviral infections needs to be investigated further due to the limited number of subjects using corticosteroids.

We conclude that this study supports the feasibility of using the throat swabs and studying the presence of adenovirus genome with the PCR method. The results suggest that adenovirus can precipitate wheezing in infancy and asthma exacerbations in asthmatic patients. Due to the ratio of adenovirus in patients both using and not using ICS, we suggest that ICS treatment has no effect on wheezing caused by adenovirus in asthmatic children.

Given that there is limited evidence on the role of adenoviruses and asthma attacks in children, the present study provides preliminary evidence on the subject. Larger studies using alternative designs need to be carried out in order to clarify the exact role of these viruses in asthma exacerbations in children.

REFERENCES