Persistence of Viruses in Upper Respiratory Tract of Children with Asthma

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Objectives: Nasopharyngeal swabs of 50 asthmatic children in the symptom-free period were examined for the presence of adenoviruses, rhinoviruses and coronaviruses. A control group of 20 healthy individuals was included in this study.
Methods: A polymerase chain reaction was used to detect adenovirus DNA and rhinovirus and coronavirus complementary DNA. The fragments of amplified genetic material were visualized with the use of agarose gel electrophoresis.
Results: Adenovirus DNA was found in 78.4% of asthmatic children, rhinovirus RNA in 32.4% and coronavirus RNA in 2.7%. Adenovirus DNA was detected in one of the 20 nasopharyngeal swabs of healthy controls; the rest of the control samples were negative.
Conclusions: The persistent presence of viruses in the upper respiratory tract of asthmatic children shows a possible connection between viral infections and asthma.   © 2000 The British Infection Society

Introduction

Asthma is the most frequent chronic respiratory disease, affecting up to 10% of children.[1,2] Asthma induced by allergens has been studied intensively and the pathogenesis is well defined. In addition to allergens, respiratory viruses have been proven to be important factors in provoking asthmatic attacks.[2–4] Virus-induced damage to the respiratory epithelium results in locally intensified activity of various cells involved in the immune response.[5,6] The presence of viruses in the respiratory tract forms a permanent source of foreign proteins, which might be the cause of subsequent pathological changes.[7] Rhinoviruses, coronaviruses, adenoviruses, respiratory syncytial virus and other paramixoviruses are most frequently presumed to be the potential cause of virus-induced asthma exacerbation.[8–10]

A vast number of virus and host characteristics dictate the type of infection, either persistent or latent, and determine host response and the resulting development of the disease.[11–13] Destruction of the infected cell or reorganization of viral antigens within the cell or on the cell membranes are commonly considered to be the consequence of virus infection. The host's immune system should finally destruct viruses.[4]

In this study nasopharyngeal swabs were taken from asthmatic children whose asthma was well controlled, and when they were at least 3 weeks free of any respiratory infections. The samples were examined for the presence of adenovirus DNA and rhinovirus and coronavirus RNA.

Patients

Fifty children, aged between 5 and 17 years with moderate asthma, were included in the study. Up to five subsequent nasopharyngeal swabs were taken from each child during the symptom-free period. For at least 3 weeks they displayed no symptoms of upper airway infections and with peak expiratory flow rates (PEFR), above 80% of their best PEFR. When the samples were taken, they had been receiving inhaled glucocorticoids (fluticasone 100 – 250 μg daily) for at least 6 months. A total of 88 samples from the asthmatic children and 20 samples from the control group (aged 5–24 years) were examined for the presence of the previously mentioned viruses.

Positive Control Viruses

To ensure a positive control, the following viruses were included in the diagnostic procedure: adenovirus type 1 and type 5 (Institute of Microbiology and Immunology,

Table I. Specific primers for adenovirus, rhinovirus and coronavirus used in PCR, their target genes and product size.

Virus	Specific primers	Target gene	Product size
Adenovirus	Outer primers as 5′ CAGCACGCCGCGGATGTCAAAGT 3 s 5′ GCCGCAGTGGTCTTACATGCACATC 3	Hexon	300 bp
	Nested primers as 5′ TTGTACGAGTACGCGGTATCCTCGCGGTC 3 s 5′ GCCACCGAGACGTACTTCAGCCTG 3		142 bp
Rhinovirus	as 5′ CGGACACCCAAAGTAG 3 s 5′ GCACTTCTGTTTCCCC 3	5 noncoding region of type 1 B	380 bp
Coronavirus	Outer primers as 5′ TGCACTAGGGGTTAATGAAGAGG 3 s 5′ GGTACTCCTAAGCCTTCTCG 3	Nucleocapsid	370 bp
	Nested primers as 5′ GACTATCAAACAGCATAGCAGC 3 s 5′ TTTGGAAGTGCAGGTGTTGTGG 3		116 bp

Medical Faculty, Ljubljana, Slovenia); rhinovirus 1B, 632, 90-05 (ATCC, USA); and human coronavirus, strain 229E, lot 5W (ATCC, USA).

Methods

Processing of clinical samples

The samples in transport medium were vigorously vortexed and subsequently centrifuged at 2000 rpm for 10 min. The pellet was resuspended in 400 µl of PBS and kept in 200 µl aliquots at −70 °C until used.

Polymerase chain reaction (PCR)

Isolation of DNA

Two hundred microlitres of cell suspension were used for the isolation of DNA using the QIAamp Blood Kit (QIAGEN Gm bH, Germany).[14] One hundred microlitres of isolated DNA were kept in an elution buffer at −20 °C, unless immediatly used for PCR.

Detection of adenovirus DNA

PCR reaction with outer primers was followed by nested PCR (n-PCR) with inner primers. Two control adenovirus types were included in each PCR reaction. PCR was carried out in a volume of 50 µl. The PCR mixture contained 5 µl of DNA preparation, $10 \times$ reaction buffer, 25 mM MgCl$_2$, 20 mM dNTP, 50 µM of each primer (TIB MOLBIOL, Berlin, Germany) (Table I), 5 U/µl of DNA Taq polymerase and DEPC water. All the reagents were provided by Perkin Elmer (New Jersey, USA). The reaction mixture was placed in a thermal cycler (Eppendorf Gradient) and heated to 93°C for 4 min. The samples were subjected to 40 cycles at 93 °C (30 s) for denaturation, at 60 °C (30 s) for annealing and at 72 °C (60 s) for elongation. In the subsequent n-PCR the pair of inner primers was used (Table I). The original DNA samples were replaced by amplicons of the first PCR.[15]

Isolation of RNA

One hundred and forty microlitres of frozen clinical material were used for isolation of RNA following RNeasy Total RNA purification Protocol (QIAGEN GmbH, Germany).[16] Fifty microlitres of RNA was eluted in DEPC water and kept at −70 °C until tested in PCR.[15]

Detection of rhinovirus and coronavirus RNA

RNA samples were reverse transcribed into complementary DNA copy (c-DNA) at 37 °C for 1 h in the 50 µl reaction mixture containing $10 \times$ reaction buffer, 25 mM MgCl$_2$, 20 mM dNTP, 20 U/µl of RNase inhibitor, 21 U/µl of AMV reverse transcriptase, 50 µM of each random primer and DEPC water. All reagents were provided by Perkin Elmer (New Jersey, USA). Five microlitres of c-DNA product were subsequently amplified in 50 µl PCR reaction mixture consisting of $10 \times$ reaction buffer, 25 mM MgCl$_2$, 20 mM dNTP, 5 U/µl of DNA Taq polymerase (all reagents provided by Perkin Elmer, New Jersey, USA) and 50 µM of each specific primer for rhinoviruses and coronaviruses (TIB MOLBIOL, Berlin, Germany) (Table I).[15] After heating the samples at 95 °C for 5 min, 35 cycles were carried out at 94 °C (60 s), 55 °C (60 s) and at 72 °C (90 s). For the detection of coronavirus RNA, n-PCR was performed using the inner pair of primers (Table I) according to the protocol used for adenovirus n-PCR.[15] In each PCR run, c-DNA of control viruses RNA was included.

Detection of amplified products: amplified products were visualized on 1.5% gel with incorporated ethidium

Table II. Number and percent values of positive, borderline and negative cases for adenovirus, rhinovirus and coronavirus NAs.

	Adenovirus				Rhinovirus				Coronavirus			
	All	+	+/−	−	All	+	+/−	−	All	+	+/−	−
Number of samples	88	69	12	7	74	24	12	38	74	2	9	63
%		78.4	13.6	8.0		32.4	16.2	51.4		2.7	12.2	85.1

bromide after electrophoresis. The amplified fragments of expected lengths (Table I) were compared to the established fragments of molecular weight markers (MWM) φX174 RF DNA/HAE III and 100 bp DNA Ladder (Life Technologies, Grand Island, USA).

Results

Presence of adenovirus DNA

PCR with the use of the outer pair of primers yielded negative results in all samples except in positive controls. However, with n-PCR, 142 bp fragment was observed in 69 out of 88 samples from asthmatic children.

Presence of rhinovirus RNA

The 380 bp fragment signifies the amplification of rhinovirus RNA. Of the 74 samples examined, 24 were positive.

Presence of coronavirus RNA

The 116 bp fragment, observed after n-PCR, indicates amplified coronavirus RNA. Positive results were obtained only in two out of 74 examined samples. A few borderline PCR results were documented for all the three viruses searched (Table II).

Coexistence of nucleic acids (NAs) of two or all three viruses searched was occasionally noticed. The follow-up of these particular samples showed that half of them harboured two or three different virus NAs persistently, while in half of them coexistence of two or three different NAs changed occasionally. Only four samples examined were truly negative for the tested viruses.

Control group

Adenovirus DNA was detected in one of the 20 samples of healthy individuals, while the rest of them were negative.

Discussion

The search for the presence of adenovirus, rhinovirus and coronavirus NAs in the upper respiratory tract of asthmatic children during the symptom-free period resulted in a high number of positive results: 78.4% of cases were positive for adenovirus DNA, 32.4% for rhinovirus RNA and 2.7% for coronavirus RNA. Johnston *et al.* found that upper respiratory tract viruses were associated with over 80% of asthma exacerbations in children.[3] However, our results relate to asymptomatic children, who were at least 3 weeks without any sign of respiratory infections. Nicholson and Beasley wanted to define the rate of virus participation in asthma exacerbation in adults. With the use of cell culture and serology, surprisingly low results were obtained.[4,17] The reason seems to be that only active infections were demonstrated. Comparing virus identification rate in children with those in adult subjects demonstrates a 50% fall in isolation rate in adults.[18] Johnston maintains that the connection between upper respiratory infections and asthma exacerbation has long been recognized, but that its importance had been underestimated in most previous studies because of deficiencies in virus-detection methods used.[19] In view of the possible presence of either active or latent infection, we think that it was well advised to use PCR in our study. Another reason for the use of this method was that coronaviruses and rhinoviruses are rather difficult to grow in cultured cells.[20,21] We have demonstrated viral DNA and RNA to be persistently present in most of asthmatic children even in the symptom-free periods. If the respiratory viruses infect only the upper respiratory tract, they affect the lower airway indirectly.[19] Gern and Fraenkel, however, confirmed that all respiratory viruses (with the exception of coronaviruses, for which no data exists) are able to infect the lower respiratory tract too.[22,23] This raises the question as to what effects, relevant to asthma, they exhibit once they get there. Due to the high viral NAs detection rate in asthmatic children compared to the lower detection rate in the control group, we can speculate that the results could be related to the disease itself or to the glucocorticoid treatment, since there is data which suggests that glucocorticoid therapy increases the titre of respiratory viruses and prolongs viral shedding.[24,25]

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