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Role of hygienic factors in the etiology of allergic disorders in children

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Background/aim: We investigated the role of body flora and chronic inflammatory infections in the etiology of allergic disorders in Turkish children.

Materials and methods: Forty pediatric asthma patients with positive skin prick tests and 40 age-matched healthy subjects with negative skin prick tests were enrolled in this cross-sectional study. Serum *H. pylori* IgG, viral hepatitis serology, IL-10, and TGF-beta levels were measured. Stool and throat cultures were taken and tested for occurrence of microorganisms.

Results: A significantly higher percentage of nonatopic subjects tested positive for anti-*H. pylori* antibodies compared to atopic subjects (60% vs. 20%). Serum IL-10 levels were also significantly higher in nonatopic subjects. No significant differences in direct microscopy and culture specimens of stools were observed. Examination of throat flora showed significantly higher occurrences of *Neisseria* and beta-hemolytic *Streptococcus* in nonatopic subjects, but higher occurrences of gram-positive bacilli in atopic subjects.

Conclusion: Higher prevalence of anti-*H. pylori* antibody and higher serum levels of IL-10 in nonatopic subjects suggest that chronic infection and inflammation may protect against atopic disease. Higher occurrences of *Neisseria* and beta-hemolytic *Streptococcus* in throat cultures from nonatopic subjects are novel findings that lend further support to the hygiene hypothesis.

Key words: Allergy, hygiene hypothesis, inflammation, microflora

1. Introduction

The prevalence of atopic disorders (atopic dermatitis, allergic rhinoconjunctivitis, allergic asthma, food allergies) has been continuously increasing over the last 20 years (1). Atopic disorders occur because of interactions between complex genetic factors, allergens, and other environmental variables (1–3). The rapid increase in atopic disorders cannot be explained by genetic changes; instead, changes in environmental conditions and lifestyle must be involved. There is a close relationship between atopy, which is the result of excess sensitivity to several allergens, and other allergic disorders, particularly in children (4).

Epidemiological studies support the view that lifestyle, environmental factors, and less exposure to infections in early life are responsible for the increase in allergic disorders. Smaller family size in industrialized societies, development of personal care and hygiene standards, vaccination, frequent use of antibiotics, less exposure to infection, and significant decrease in parasitic infections may be associated with the increase in inappropriate immune responses against nonthreatening substances. This view is known as the hygiene hypothesis, which asserts that less exposure to microorganisms that stimulate the immune system (particularly in early life) may result in the development of atopy (5).

Several studies have shown an inverse relationship between infection and allergic disorder. Fewer intestinal helminth (*Trichuris trichiura*), hepatitis A virus (HAV), and protozoa (*T. gondii*) infections and lower bacterial (*Helicobacter pylori*) load have been shown to be related to the increased frequency of atopic disease (5–7). Some findings also suggest that measles, HAV, and tuberculosis infections prevent atopic disorders (5).

Intestinal flora has been shown to regulate immune system maturation in various studies (8,9). The differences in indigenous intestinal flora were proposed to affect the maturation of the immune system in early childhood (9).

The role of *Mycobacterium* in allergic disorders has also been investigated (10,11). Mycobacterial antigens have been shown to directly suppress the allergic response

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and this suppression was dependent on the release of the antiinflammatory cytokines transforming growth factor-beta (TGF-beta) and interleukin-10 (IL-10). These cytokines have also been shown to increase in chronic helminth infections and to suppress immunological responses (12).

In this study we compare evidence of exposure to pathologic microbes, levels of antiinflammatory cytokines, and occurrence of microorganisms in stool and throat cultures from atopic and nonatopic children seen at an allergy outpatient clinic with the goal of understanding the role of body flora and infection in the etiology of allergic disorders.

2. Materials and methods

2.1. Study subjects

This study included 40 atopic children who had been diagnosed with bronchial asthma and who had a positive skin prick test and 40 nonatopic healthy children (no asthma and negative skin prick test) who were seen at the allergy outpatient clinic. Skin prick tests were done on the volar part of the front arm. Alyostal prick tests manufactured by ALK were used. Reactions to house dust mites, fungi, animal epithelium and hair, grass pollens, tree pollens, grain pollens, and weeds were measured routinely. Other allergens, such as food and latex, were tested if a subject's medical history suggested possible allergy to these substances. Hyperemia greater than 3 mm and greater than the negative control was considered as positive. Subjects were diagnosed with asthma based on history of respiratory symptoms such as wheeze, shortness of breath, chest tightness, and cough that varied over time and in intensity, together with variable expiratory airflow limitation, positive response to bronchodilator medications, increased IgE levels, and positive skin prick test. The parents or the legal guardians provided informed consent and the study was approved by the local institutional review boards.

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2.2. Serum-based assays for hepatitis and *H. pylori* exposure and cytokine levels

Blood samples were drawn and serum was prepared. Anti-HAV antibodies (IgM and IgG), hepatitis B virus surface antigen (HbsAg), and anti-HCV antibodies were measured using specific ELISA kits (Advia Centaur XP Immunoassay System, Siemens). Specific ELISA kits were also used to measure serum levels of anti-*H. pylori* antibody (*H. pylori* IgG ELISA kit, Trinity Biotech TM, USA, Ref. No: 2346400), TGF-beta 1 (Bender Med Systems, Austria, Cat. No: BMS249/2), and IL-10 (BioSource International, USA, Ref. No: 2346400). ELISA assays were scored using an ELX 800 universal microplate reader (Bio-Tec Instruments, Inc.).

2.3. Detection and identification of microorganisms in stool and throat cultures

Throat and stool culture specimens were collected from the study subjects. None of the study subjects were using antibiotics before collection of samples. Throat specimens were plated on one EMB agar plate to propagate gram-negative bacteria and two sheep blood agar plates. One blood agar plate was incubated in anaerobic conditions and the other in aerobic conditions. Stool specimens were first examined by direct microscopy, after mixing them with one drop of physiological saline, for the presence of parasites and leukocytes. Stool specimens were then plated on Schadler agar for propagation of anaerobic gram-negative bacteria, Columbia CNA agar for propagation of anaerobic gram-positive bacteria, methicillin-resistant *Staphylococcus aureus* (MRSA) agar including 0.3% potassium for propagation of *Lactobacillus* and *Streptococcus*, and Sabouraud dextrose agar for propagation of fungi. Types and subtypes of bacteria that reproduced were determined.

2.4. Statistical analysis

Analysis of data was performed using SPSS 15.0 (SPSS Inc., Chicago, IL, USA). Level of significance was set at $P < 0.05$ for all variables. The normality of distribution was assessed by Kolmogorov–Smirnov test. The Student t-test was used for variables with normal distribution. The Mann–Whitney U test was utilized comparing groups without normal distribution. The Fisher exact chi-square test was used for comparison of two groups in examination of stool and throat flora. HbsAg, anti-HAV, anti-HCV, and anti-*H. pylori* antibody levels were also compared using the Fisher exact chi-square test.

3. Results

3.1. Characteristics of study subjects

Characteristics of study subjects are shown in Table 1. The mean age of subjects was 10.38 ± 2.18 years (range: 7–15 years). No difference in age and sex distribution was recorded between the patient and control groups ($P > 0.05$).

3.2. Comparison of serum antibodies indicative of pathogen exposure in atopic and nonatopic subjects

We measured the serum levels of antibodies to hepatitis A, hepatitis C, HbsAg, and *H. pylori* as indicators of exposure to pathogenic microorganisms and compared the values from atopic and nonatopic subjects (Table 2). While a higher percentage of nonatopic subjects tested positive for anti-HAV antibodies compared to atopic subjects (32.5% vs. 17.5%), this difference was not statistically significant. Only one atopic subject tested positive for HBs, with no significant difference between atopic and nonatopic subjects. The same was true for HCV antibodies. However, a significantly higher percentage of nonatopic subjects

Table 1. Characteristics of study subjects.

	Number	Sex, female/male	Age (years)
Atopic group	40	15 / 25	10.38 ± 2.12
Nonatopic group	40	21 / 19	10.38 ± 2.25

Table 2. Serum antibodies indicative of pathogen exposure in atopic and nonatopic subjects.

	Atopic (n = 40)		Nonatopic (n = 40)		
	+	-	+	-	
Anti-HAV (total)	7 (17.5%)	33 (82.5%)	13 (32.5%)	27 (67.5%)	0.196
HbsAg	1 (2.5%)	39 (97.5%)	0	40 (100.0%)	NC
Anti-HCV	1 (2.5%)	39 (97.5%)	0	40 (100.0%)	NC
	Atopic (n = 40)		Nonatopic (n = 20)		
	+	-	+	-	P-value
Anti-*H. pylori*	8 (20.0%)	32 (80.0%)	12 (60.0%)	8 (40.0%)	0.012

HAV: Hepatitis A virus; HbsAg: hepatitis B surface antigen; HCV: hepatitis C virus; NC: not calculated.

tested positive for anti-*H. pylori* antibodies compared to atopic subjects (60% vs. 20%) (P = 0.012).

3.3. Comparison of serum cytokine levels in atopic and nonatopic subjects

We also measured serum levels of the cytokines IL-10 and TGF-beta, both of which have been shown to suppress immune function (Table 3). The average serum IL-10 concentration was significantly higher in nonatopic subjects compared to atopic subjects (27.43 ± 28.92 pg/mL vs. 5.61 ± 2.46 pg/mL) (P = 0.001). Average serum TGF-beta levels were within the normal range for both atopic and nonatopic subjects, with no significant difference between them.

3.4. Comparison of microorganisms in stool samples from atopic and nonatopic subjects

Microscopic examination of stool samples from atopic and nonatopic subjects showed no leukocytes in any samples. No evidence of parasites was seen in samples from atopic and nonatopic subjects, but *Giardia* cysts were observed in 2 of the 40 samples from atopic subjects. This difference in parasite prevalence was not significant.

Stool samples were cultured on various media to identify microorganisms present. No significant differences were found between stool samples from atopic and nonatopic subjects with regard to the occurrence of *Candida* or *E. coli*, *Corynebacterium*, *Enterococcus*, *Streptococcus*, and other bacteria (Table 4). *E.coli* was the most frequently observed bacteria in stool samples from both the atopic and nonatopic subjects, occurring in 97.5% of samples from atopic subjects and 95% of samples from nonatopic subjects (Table 4).

3.5. Comparison of bacteria in throat cultures from atopic and nonatopic subjects

Throat cultures from atopic and nonatopic subjects were examined for the presence of bacteria. No statistically significant differences were observed between atopic and nonatopic groups in occurrence of *Streptococcus*, coagulase-negative *Staphylococcus*, diphtheroids, *Pneumococcus*, or *Klebsiella* in throat cultures (Table 5). *Neisseria* and beta-hemolytic *Streptococcus* were observed in a significantly higher percentage of samples from nonatopic subjects compared to atopic subjects, while *Bacillus* was observed

Table 3. Cytokine levels in atopic and nonatopic subjects.

Atopic (n = 40)	Nonatopic (n = 40)	P-value	
IL-10 (pg/mL)	5.61 ± 2.46	27.43 ± 28.92	0.001
TGF-beta (pg/mL)	8.26 ± 5.11	7.75 ± 4.69	0.419

Normal IL-10 serum level: 0–3.3 pg/mL.
Normal TGF-beta serum level: 0.47–30 ng/mL.

Table 4. Microorganisms in stool samples from atopic and nonatopic subjects.

	Atopic (n = 40)		Nonatopic (n = 40)		P-value
	+	-	+	-	
Gram-negative bacilli					
E. coli	39 (97.5%)	1 (2.5%)	38 (95.0%)	2 (5.0%)	1.00
Bacteroides	6 (15.0%)	34 (85.0%)	6 (15.0%)	34 (85.0%)	1.00
Gram-positive bacilli					
Lactobacillus	16 (40.0%)	24 (60.0%)	9 (22.5%)	31 (77.5%)	0.147
Corynebacterium	8 (20.0%)	32 (80.0%)	17 (42.5%)	23 (57.5%)	0.053
Anaerobic diphtheroids	0	40 (100.0%)	3 (15.0%)	37 (92.5%)	0.241
Gram-positive cocci					
Enterococcus	24 (60.0%)	16 (40.0%)	20 (50.0%)	20 (50.0%)	0.500
Coagulase-negative *Staphylococcus*	3 (7.5%)	37 (92.5%)	6 (15.0%)	34 (85.0%)	0.481
Streptococcus	6 (15.0%)	34 (85.0%)	11 (27.5%)	29 (72.5%)	0.274
Yeast					
Candida	3 (7.5%)	37 (92.5%)	4 (10.0%)	36 (90.0%)	1.00

Table 5. Bacteria in throat cultures from atopic and nonatopic subjects.

	Atopic (n = 40)		Nonatopic (n = 40)		P-value
	+	-	+	-	
Gram-negative bacilli					
Klebsiella	4 (10.0%)	36 (90.0%)	2 (5.0%)	38 (90.0%)	0.675
Gram-positive bacilli					
Bacillus	6 (15.0%)	34 (85.0%)	0	40 (100.0%)	0.026
Lactobacillus	1 (2.0%)	39 (97.5%)	0	40 (100.0%)	NC
Anaerobic diphtheroids	2 (5.0%)	38 (95.0%)	3 (7.5%)	37 (92.5%)	1.00
Gram-negative cocci					
Neisseria	26 (65.0%)	14 (35.0%)	40 (100.0%)	0	0.001
Gram-positive cocci					
Viridans *Streptococcus*	34 (85.0%)	6 (15.0%)	37 (92.5%)	3 (7.5%)	0.481
Coagulase-negative *Staphylococcus*	27 (67.5%)	13 (32.5%)	20 (50.0%)	20 (50.0%)	0.173
Pneumococcus	2 (5.0%)	38 (95.0%)	5 (12.5%)	35 (87.5%)	0.432
Beta-hemolytic *Streptococcus*	0	40 (100.0%)	14 (35.0%)	26 (65.0%)	0.001

NC: Not calculated.

in a significantly higher percentage of samples from atopic subjects compared to nonatopic subjects. Viridans streptococci were the most frequently observed bacteria in throat cultures from atopic and nonatopic subjects, occurring in 85% of samples from atopic subjects and 92.5% of samples from nonatopic subjects.

4. Discussion

We found that anti-*H. pylori* antibodies were significantly higher in nonatopic subjects compared to atopic and that, while not a significant difference, anti-HAV antibodies tended to be higher in nonatopic compared to atopic subjects. Several studies have shown that infections that are transmitted through ingestion, such as HAV, *T. gondii*, and *H. pylori*, decrease the risk of atopy (5–7). In a study of 240 atopic and 240 nonatopic young adults from Italy, Matricardi et al. (13) found a lower prevalence of HAV and *H. pylori*, as well as *T. gondii*, in atopic subjects. The incidence of atopy was lowest in subjects who had been exposed to more than one of these agents. Thus, infection via ingestion seems to effectively protect against the development of allergy and atopic disorders.

Many studies have focused on the effect of chronic parasite infections on the prevalence of atopy. Scrivener et al. conducted a broad-range study in urban and rural areas of Ethiopia and found that parasite infections were more common in rural areas and asthma prevalence was significantly lower in rural areas (14). Other studies support the idea that chronic parasite infections lower the risk of atopy (15–17). Unfortunately, no conclusion on the association of parasite infection and atopy could be made from our study since none of the subjects were found to have parasitic infections. This may be due to the fact that all the subjects in the present study lived in urban areas.

Release of antiinflammatory cytokines IL-10 and TGF-beta occur as the result of continuous antigenic stimulation in cases of chronic inflammatory infections (HAV, *H. pylori*, *Toxoplasma*) and chronic parasitic infections (12). These cytokines play a key role in the development of tolerance against environmental allergens. In the present study we found that serum IL-10 levels were significantly higher in nonatopic subjects compared to atopic subjects. Similar results were found in a study of Danish babies (18). However, Corne et al. (19) found higher levels of IL-10 in nasal lavage from atopic subjects compared to nonatopic subjects.

TGF-beta has also been implicated in the development of atopic disease. An in vitro study showed that TGF-beta inhibits the release of the proinflammatory cytokines gamma-interferon and alpha-TNF in keratinocyte cells (20). Lower expression of the TGF-beta effector molecule Smad7 was found in asthma patients compared to controls (21). In the present study, however, we did not find a significant difference in serum TGF-beta levels between atopic and nonatopic subjects.

In the present study we did not observe significant differences in direct microscopy and culture specimens of stools from atopic and nonatopic subjects. We found significant differences in throat flora between atopic and nonatopic subjects. Unlike intestinal microflora, throat microflora has not been well studied in the context of allergic disease. Thus, these findings are quite novel. We found that gram-positive beta-hemolytic *Streptococcus* and gram-negative *Neisseria* were significantly more prevalent in throat cultures from nonatopic subjects compared to atopic subjects. The high prevalence of gram-negative *Neisseria* is particularly interesting. Heterogeneous lipopolysaccharide (LPS) in the cell wall of gram-negative bacteria is one of the most important immune-stimulating molecules. LPS induces IL-12 production and a proinflammatory Th1 response. It stimulates type 1 immunity through molecular mechanisms such as inhibition of Th2 response and allergic inflammation (17). Our finding that throat cultures from nonatopic subjects had a higher occurrence of *Neisseria* and beta-hemolytic *Streptococcus* suggests that exposure to these microorganisms may protect against atopic disease.

In conclusion, the prevalence of anti-*H. pylori* antibodies and serum levels of antiinflammatory cytokine IL-10 were higher in nonatopic subjects than in atopic subjects, suggesting that chronic infection and inflammation may protect against atopic disease. Examination of throat wall flora showed significantly higher occurrence of the LPS-producing gram-negative bacteria *Neisseria* and gram-positive beta-hemolytic *Streptococcus* in nonatopic subjects compared to atopic subjects. These novel findings lend further support to the hygiene hypothesis.

References

1. Sengler C, Lau S, Wahn U, Nickel R. Interactions between genes and environmental factors in asthma and atopy: new developments. Respir Res 2002; 3: 7-37.
2. Vercelli D. The functional genomics of CD14 and its role in IgE responses: an integrated view. J Allergy Clin Immunol 2002; 109: 14-21.
3. Huang SK, Marsh DG. Genetics of allergy. Ann Allergy 1993; 70: 347-359.
4. Taylor AJN. Asthma and allergy. BMJ 1998; 316: 997.
5. Yazdanbakhsh M, Kremsner P, Van Ree R. Allergy, Parasites and the hygiene hypothesis. Science 2002; 296: 490-494.
6. Loke P, Lim YAL. Helminths and the microbiota: parts of the hygiene hypothesis. Parasite Immunol 2015; 37: 314-323.
7. Kondrashova A, Seiskari T, Ilonen J, Knip M, Hyöty H. The 'Hygiene hypothesis' and the sharp gradient in the incidence of autoimmune and allergic diseases between Russian Karelia and Finland. APMIS 2013; 121: 478-93.

8. Sepp E, Julge K, Vasar M, Naaber P, Björksten B, Mikelsaar M. Intestinal microflora of Estonian and Swedish infants. Acta Paediatr 1997; 86: 956-961.

9. Björkstén B, Naaber P, Sepp E, Mikelsaar M. The intestinal microflora in allergic Estonian and Swedish 2 year-old children. Clin Exp Allergy 1999; 29: 342-346.

10. Barlan I, Tükenmez F, Bakır M. Effect of BCG Vaccination on T Helper Cell Cytokine Response in Atopic Children. Ankara, Turkey: TÜBİTAK Project No. SBAG-1432 (in Turkish).

11. Hadley EA, Smillie FI, Turner MA, Custovic A, Woodcock A, Arkwright PD. Effect of *Mycobacterium vaccae* on cytokine responses in children with atopic dermatitis. Clin Exp Immunol 2005; 140: 101-108.

12. van den Biggelaar AH, van Ree R, Rodrigues LC, Lell B, Deelder AM, Kremsner PG, Yazdanbakhsh M. Decreased atopy in children infected with *Schistosoma haematobium*: a role for parasite-induced IL-10. Lancet 2000; 356: 1723-1727.

13. Matricardi PM, Rosmini F, Riondino S, Fortini M, Ferrigno L, Rapicetta M, Bonini S. Exposure to foodborne and orofaceal microbes versus airborne viruses in relation to atopy and allergic asthma: epidemiological study. BMJ 2000; 320: 412-417.

14. Scrivener S, Yemanerberhan H, Zebenigus M, Tilahun D, Girma S, Ali S, McElroy P, Custovic A, Woodcock A, Pritchard D et al. Independent effects of intestinal parasite infection and domestic allergen exposure on risk of wheeze in Ethiopia: a nested case control study. Lancet 2001; 358: 1493-1499.

15. Salminen S, Isolauri E, Onella T. Gut flora in normal and disordered states. Chemothrerapy 1995; 41: 5-15.

16. Adlerberth I, Carlsson B, de Man P, Jalil F, Khan SR, Larsson P, Mellander L, Svanborg C, Wold AE, Hanson LA. Intestinal colonization with Enterobacteriaceae in Pakistani and Swedish hospital-delivered infants. Acta Paediatr Scand 1991; 80: 602-610.

17. Matricardi PM, Bonini S. High microbial turnover rate preventing atopy: a solution to inconsistencies impinging on the hygiene hypothesis. Clin Exp Allergy 2000; 30: 1506-1510.

18. van der Velden VH, Laan MP, Baert MR, de Waal Malefyt R, Neijens HJ, Savelkoul HF. Selective development of a strong Th2 cytokine profile in high-risk children who develop atopy: risk factors and regulatory role of IFN-gamma, IL-4 and IL-10. Clin Exp Allergy 2001; 31: 997-1006.

19. Corne JM, Lau L, Scott SJ, Davies R, Johnston SL, Howarth PH. The relationship between atopic status and IL-10 nasal lavage levels in the acute and persistent inflammatory response to upper respiratory tract infection. Am J Respir Crit Care Med 2001; 163: 1101-1107.

20. Sumiyoshi K, Nakao A, Setoguchi Y, Tsuboi R, Okumura K, Ogawa H. TGF-beta/Smad signaling inhibits IFN-gamma and TNF-alpha induced TARC (CCL17) production in HaCaT cells. J Dermatol Sci 2003; 31: 53-58.

21. Nakao A, Sagara H, Setoguchi Y, Okada T, Okumura K, Ogawa H, Fukuda T. Expression of Smad7 in bronchial epithelial cells is inversely correlated to basement membrane thickness and airway hyperresponsiveness in patients with asthma. J Allergy Clin Immunol 2002; 110: 873-878.