Scientific Article

Human Leukocyte Antigen Class II Alleles and Dental Caries in a Child Population

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Abstract: **Purpose:** The purpose of this study was to investigate a possible relationship between human leukocyte antigens (HLAs) DRB1 and DQB1, dental caries, and colonization by mutans streptococci (MS) in children. **Methods:** Sixty children were clinically examined for caries in accordance with World Health Organization criteria and methods. Thereafter, subjects were assigned into 2 groups: (1) high-caries children (dft and DMFT ≥5); and (2) caries-free children (dft and DMFT=0). Fresh saliva samples were collected and tested for mutans streptococci, after which the subjects were placed into 2 groups, having either high (≥10^5 colony-forming units [CFU]/mL saliva) or low (<10^5 CFU/mL saliva) numbers of micro-organisms in saliva. The polymerase chain reaction/sequence specific primer method was used to determine HLA DNA typing from fresh blood samples. **Results:** There was no significant difference between the frequency of HLA alleles in high-caries and caries-free subjects. Although chi-square test suggested an association for HLA-DRB1*01 and HLA-DQB1*03 with the salivary numbers of MS (P=.026 and P=.009, respectively), these could not be confirmed by logistic regression analysis (P=.188 and P=.101, respectively). **Conclusions:** The results obtained fail to establish an association between human leukocyte antigen alleles DRB1 and DQB1 and salivary numbers of MS in the selected child population. (Pediatr Dent 2008;30:154-9) Received April 4, 2007 / Last Revision August 10, 2007 / Revision Accepted August 14, 2007.

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Dental caries is a bacterial infection caused by acidogenic and aciduric bacteria colonizing in the oral cavity.[1,2] Being one of the most prevalent chronic diseases worldwide, individuals are susceptible to dental caries during their entire lifetime.[1] Mutans streptococci (**MS**), *Lactobacillus acidophilus*, and *Lactobacillus casei* contribute to the cariogenic potential of dental plaque.[3,4] The presence of MS in the plaque or saliva of young, caries-free children appears to be associated with a considerable increase in caries risk.[5] Saliva, oral hygiene, and diet are well-known factors that affect the colonization of *Streptococcus mutans*. They do not, however, fully explain the interindividual differences in colonization levels,[6-11] and the essential role of MS in the caries process is not proved.[6] Therefore, it is important to identify factors that may influence their colonization. If the caries susceptibility is in part influenced by the oral microbiota, then one's immune response capability may also be modulating this process,[12] and there are data to support an immunological basis for caries resistance. Caries-free individuals have been observed to increase serum antibody titers to streptococcal antigens.[13] Moreover, the T-lymphocytes from these individuals have an enhanced ability to proliferate upon exposure to streptococcal antigens.[14] Caries-resistant subjects have also been observed to generate greater T-helper cell activity to a lower dose of purified streptococcal antigen compared to caries-prone individuals.[15]

The term **HLA** refers to the human leukocyte antigen system, which is controlled by genes on the short arm of chromosome 6. The HLA loci are part of the genetic region known as the major histocompatibility complex (**MHC**). The MHC has genes (including HLA) that are integral to normal functioning of the immune response.[16,17] The essential role of HLA antigens lies in the control of self-recognition and, thus, defense against micro-organisms. Based on the structure of the antigens produced and their function, there are 2 classes of HLA antigens, termed HLA Class I and Class II. The remaining section, sometimes known as Class III, contains loci responsible for complement, hormones, intracellular peptide processing, and other developmental characteristics. Thus, the Class III region is not actually a part of the HLA complex, but is located within the HLA region, because its components are either related to the functions of HLA antigens or are under similar control mechanisms to the HLA genes.

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Routine Tissue Typing identifies the alleles at 3 HLA Class I loci (HLA-A, B, and C) and 3 Class II loci (HLA-DR, DP, and DQ). More diseases, mainly autoimmune, have been shown to be associated with alleles of the HLA region than any other genetic region.[18] The penetrance of disease in individuals carrying the associated HLA allele is often low, however, which can be attributed to environmental factors and to the influence from other genes.[18] HLA-associated diseases have been identified in virtually every major organ system, and most of them are regarded as autoimmune diseases.[19] Several population studies are available suggesting the association of HLAs in more than 40 diseases.[19] Diseases with a strong link to certain HLA genes include type I diabetes,[20,21] rheumatoid arthritis,[22,23] ankylosing spondylitis,[24,25] and celiac disease.[26] Infectious diseases—such as malaria,[27] tuberculosis,[28,29] leprosy,[30] hepatitis B,[31] hepatitis C,[32] and HIV/AIDS[33,34]—are also associated with increased or decreased susceptibility with HLA.[19,35]

The intricate immune processes that regulate the production of antibodies in saliva are influenced by the HLA molecules on the immune cells.[36,37] Lehner et al[15] have reported that lymphocytes from caries-resistant humans released helper factor (ie, cytokines) on the addition of 1 to 10 ng of the purified streptococcal antigen (**SA**) I/II, whereas lymphocytes from caries-susceptible subjects required 1,000 ng SA I/II for a similar release of helper factor.[38] This difference in dose response was analyzed in relation to the HLA antigens, and a significant relationship was observed for the HLA-DR4 antigen. The serologically defined DR4 group can genetically be divided into at least 19 DRB1* subgroups.[37] The subgroups reflect the amino acid variation of the peptide-binding groove in the HLA-DR molecule. As the immune response depends on the affinity between the antigenic peptide and the HLA Class II molecule, a search for an association between HLA and antibody response may preferably be sought on a subgroup level.[39,40]

In light of these observations, the alleles of HLA Class II genes, which are engaged in the wide range of immune response, may play a role in oral accumulation of MS. Consequently, the aim of this study was to investigate a possible relationship between HLA antigens DRB1 and DQB1, dental caries, and colonization by MS in children.

Methods

Sixty 5- to 12-year-old Caucasian children (29 females and 31 males) were recruited among patients attending the department of pediatric dentistry Gulhane Medical Academy, Ankara, Turkey. After explaining the study protocol in detail to parents and legal guardians, their informed consent was obtained to allow for the experimental procedure. Both the consent form and the research protocol were approved by the Institutional Human Subject Review Committee at the Gulhane Medical Academy. Patients who had taken antibiotics or fluoride supplements or had undergone preventive and/or restorative treatments within the past 3 months were excluded.

Clinical examination of dental caries was carried out using a mirror and explorer, in accordance with World Health Organization criteria and methods.[41] The total number of decayed, missing, and filled permanent and primary teeth (DMFT, dmft) were recorded in each patient without radiographic exams. Accordingly, subjects were assigned into 2 groups: (1) group 1=high-caries children (dft and DMFT ≥5); and (2) group 2=caries-free children (dft and DMFT=0).

Saliva was collected for each subject at the same clinical examination appointment. The procedure was carried out between 9:30 a.m. and 12 noon, and each patient was instructed not to eat or drink for 1 hour preceding an appointment. Before starting saliva collection, each patient was given a simple explanation as to the nature and reason for the tests. They were seated in a dental chair in a quiet surgery setting and given time to acclimate to the environment. Approximately 5 mL of whole paraffin-stimulated fresh saliva was collected from each patient and immediately transferred to the laboratory inside sterile disposable plastic containers. Each sample was subjected to 1/10, 1/100, 1/1,000 dilution. Diluted samples were directly transferred to 5% blood sheep agar and mitis-salivarius agar (Biological Labs Inc, Baltimore, Md) and incubated for 48 hours at 37°C in a CO_2 incubator. Thereafter, micro-organisms were identified using API ID 32 Strep (Biomerieux, France) and calculated as colony forming units (**CFUs**). The subjects were placed into 2 groups, having either high or low numbers of micro-organisms in saliva samples (Table 1). Cutoff points for high numbers were defined as ≥105 CFU per mL of saliva for MS.[42]

Table 1.   COMPARISON BETWEEN LOW AND HIGH NUMBERS OF MUTANS STREPTOCOCCI (MS) IN RELATION TO MEANS OF DFT AND DMFT

MS (CFU/mL)	Low(<10⁵)	High (≥10⁵)	P-value
No. of subjects	41	19	
Gender (male/female)	21/20	11/8	.65
Age (mean±SD)	9.56±1.59	10.11±1.37	.21
dft (mean±SD)	1.85±2.99	3.42±2.17	.045
DMFT (mean±SD)	1.44±2.61	5.05±4.12	<.001

DNA extraction and polymerase chain reaction (**PCR**) amplifications were performed according to the manufacturer's instructions of the kits used. DNA extraction was carried out by the conventional phenol-chloroform method from 2 to 5 mL fresh whole blood. The PCR/sequence specific primer (PCR/ **SSP**) method HLA SSP Typing Kit (R.O.S.E. Europe GMBH, Frankfurt, Germany) was used to determine HLA typing. Accordingly, DNA sequences are amplified with the help of specific primers that bind only to a complementary DNA sequence. Only if the primer matches exactly with the target DNA can this DNA be amplified very efficiently, as opposed to primers with mismatches. The HLA SSP kit primers are designed to match only completely with one single allele or allele group.

A dried primer stock solution consisting of an HLA specific primer mix (ie, allele and group specific primers and internal positive control primer pairs) was aliquoted in 0.2 mL PCR tubes. To check PCR conditions, the internal positive control primer pairs that amplify the human growth hormone gene segments were used in each specimen. The PCR master mix contained 0.4 U/ul Taq polymerase, 200 uM dNTPs, 50 mM KCl, 1.5 mM MgCl$_2$, 10 mM Tris-HCl (pH 8.3), 0.001% w/v gelatin, 5% glycerol, and 100 ug/mL cresol red at final concentration. The amplification reaction contained 2 uL template DNA (20-100 ng/mL), 3 uL PCR master mix, and 5 uL nuclease-free dH2O for 1 SSP reaction. PCR cycling parameters included:

1. denaturation at 94ºC for 2 minutes;
2. 1 cycle, denaturation at 94ºC for 10 seconds, annealing, and extension at 65ºC for 60 seconds;
3. 10 cycles, denaturation at 94ºC for 10 seconds, annealing at 61ºC for 50 seconds, extension at 72ºC for 30 seconds;
4. 20 cycles, as per the manufacturer's descriptions (HLA DR&DQ Combi SSP, Olerup SSP AB, Saltsjöbaden, Sweden).

PCR products were electrophoresed on 2% agarose gel. Presence and relative lengths of the specific PCR products were interpreted with Helmberg-Score interpretation software (GenoVision Inc, West Chester, Pa).

Statistical analysis. SPSS statistical software (v. 11.5, SPSS Inc, Chicago, Ill) was utilized for statistical analysis of the data. Mean age (±SD) and dft-DMFT were calculated and compared with the salivary numbers of MS. The significance of the difference of means was evaluated with chi-square tests or Student's *t* test at a significance level of 5%. When appropriate, the frequencies of alleles and haplotypes were compared between high and low numbers of salivary oral micro-organisms using Fisher's exact test (*P*=.05) or chi-square test. Backward logistic regression was used to evaluate the associations between caries and HLA groups and between the MS levels and HLA groups. Covariance analysis was used to assess the variables affecting dft and DMFT levels.

Results

The average patient age was 9.73±1.54 (SD) years. Of the 60 samples tested for MS, 41 had low and 19 had high salivary numbers (Table 1). The low and high numbers for MS were not significantly related to the mean age and gender of subjects (*P*>.05). The mean dft and DMFT were significantly higher in the high-number group (≥105 CFU /mL saliva) for MS than that of the low-number group (<105 CFU/mL saliva; *P*=.045 and *P*<.001, respectively; Table 1).

A possible relationship between dft/DMFT and MS was assessed by covariance analysis. Accordingly, dft and DMFT were used as dependent variables, MS levels were used as independent variables, and age was used as a covariate. Significant

relationships were found between levels of MS and both dft (f=7.54, *P*=.008) and DMFT (f=14.96, *P*=.001).

Frequencies of HLA-DRB1 and HLA-DQB1 alleles in the low-number and high-number groups of MS are presented in Table 2. HLA-DRB1*01 and HLA-DQB1*03 were associated with the salivary numbers of MS (*P*=.026 and *P*=.009, respectively). When the data were analysed by logistic regression, however, the associations for HLA-DRB1*01 and HLA-DQB1*03 could not be confirmed (Table 3).

The frequency of HLA-DRB1 and HLA-DQB1 alleles and caries are presented in Table 4. There was no difference between frequencies of HLA type in the high-caries and caries-free subjects (*P*>.05).

Discussion

MS are found in almost all individuals, though there are large differences in the colonization levels between them. These differences are not readily explained, while several factors belonging to the individual (diet, oral hygiene, fluoride exposure, etc) are believed to influence the colonization. Although the

Table 2.	FREQUENCIES (%) OF HLA-DRB1 AND HLA-DQB1 ALLELES IN LOW AND HIGH SALIVARY NUMBERS OF MUTANS STREPTOCOCCI (MS)*			
	MS (CFU/mL)			
	Total (+) N=60	Low(<10^5) N=60	High (≥10^5) N=60	*P*-value
	N(%)	N(%)	N(%)	
DRB1*01	17 (28)	8 (20)	9 (47)	.03
DRB1*02	1 (2)	1 (2)	0 (0)	<.001
DRB1*03	10 (17)	6 (15)	4 (21)	.71
DRB1*04	13 (22)	10 (24)	3 (16)	.52
DRB1*07	6 (10)	4 (10)	2 (11)	<.001
DRB1*08	8 (13)	5 (12)	3 (16)	.70
DRB1*09	4 (7)	1 (2)	3 (10)	.09
DRB1*10	4 (7)	2 (5)	2 (11)	.59
DRB1*11	17 (28)	13 (32)	4 (21)	.39
DRB1*12	3 (5)	2 (5)	1 (5)	<.001
DRB1*13	12 (20)	9 (22)	3 (16)	.74
DRB1*14	9 (15)	6 (15)	3 (16)	<.001
DRB1*15	5 (8)	4 (10)	1 (5)	<.001
DRB1*16	7 (12)	7 (17)	0 (0)	.09
DQB1*02	25 (42)	17 (42)	8 (42)	.97
DQB1*03	42 (70)	33 (81)	9 (47)	.01
DQB1*04	12 (20)	6 (15)	6 (32)	.17
DQB1*05	24 (40)	14 (34)	10 (53)	.17
DQB1*06	9 (15)	5 (12)	4 (21)	.45

* *P*-values were computed from the chi-square test; (N)=no. of subjects presented in Table 1.

Table 3.	RESULTS OF THE LOGISTIC REGRESSION ANALYSIS FOR HLA-DRB1*01 AND HLA-DQB1*03 ALLELES			
	MS*		Caries	
	OR (95% CI)	P-value†	OR (95% CI)	P-value
Mutans			20.57 (3.55-119.21)	.001
Caries	19.63 (3.46-111.48)	.001		
DRB1*01	2.05 (0.42-9.88)	.373	1.74 (0.36-8.39)	.49
DQB1*03	0.335 (0.08-1.48)	.149	0.62 (0.13-2.91)	.62
Age	1.33 (0.81-2.18)	.266	0.66 (0.41-1.05)	.08

* Mutants streptococci

† P-values were computed from the chi-square test; (N)=no. of subjects presented in Table 1.

Table 4.	FREQUENCIES (%) OF HLA-DRB1 AND HLA-DQB1 ALLELES AND CARIES STATUS*			
	CARIES			
	High caries N=30	Caries-free N=30	Total (+) N=60	P-value
	N(%)	N(%)	N(%)	
DRB1*01	11(37)	6 (20)	17 (28)	.15
DRB1*02	1 (3)	0 (0)	1 (2)	<.001
DRB1*03	5 (17)	5 (17)	10 (17)	<.001
DRB1*04	7 (23)	6 (20)	13 (22)	.75
DRB1*07	2 (7)	4 (13)	6 (10)	.67
DRB1*08	4 (13)	4 (13)	8 (13)	<.001
DRB1*09	1 (3)	3 (10)	4 (7)	.61
DRB1*10	3 (10)	1 (3)	4 (7)	.61
DRB1*11	6 (20)	11 (37)	17 (28)	.15
DRB1*12	3 (10)	0 (0)	3 (5)	.24
DRB1*13	6 (20)	6 (20)	12 (20)	<.001
DRB1*14	3 (10)	6 (20)	9 (15)	.47
DRB1*15	3 (10)	2 (7)	5 (8)	<.001
DRB1*16	3 (10)	4 (13)	7 (12)	<.001
DQB1*02	13 (43)	12 (40)	25 (42)	.38
DQB1*03	18 (60)	24 (80)	42 (70)	.09
DQB1*04	5 (17)	7 (23)	12 (20)	.52
DQB1*05	13 (43)	11 (37)	24 (40)	.60
DQB1*06	7 (23)	2 (7)	9 (15)	.15

* N=no. of subjects presented in Table 1.

present study's results confirm the well-known relationship between dental caries and salivary numbers of MS,[43-46] this study's main purpose was to investigate a possible relationship between HLA-DRB1 and HLA-DQB1 alleles and dental caries as well as colonization by MS. To the best of our knowledge, this is the first study conducted in a child population to search for a possible association between MHC genes and MS levels.

We did not observe an association between any of the studied alleles with the DMFS index. This finding is line with previous studies that failed to observe an association between dental caries and HLA-DRB1 alleles.[12,47] This is not surprising, considering that caries formation depends on many factors besides the levels of cariogenic organisms and immune responsiveness, including diet, tooth anatomy, and overall health.[10,48,49]

Lehner et al[15] analyzed the distribution of HLA-DR antigens in a group of 24 individuals with either high or low DMFS indices. They showed that HLA DRw6-1,2,3 had a significant relationship with the DMFS index and a low-dose response to MS antigens. HLA-DR4 did not demonstrate the same relationship to caries incidence. A similar study was conducted by de Vries et al[47] on military recruits matched for all criteria other than caries-free and caries-active, but no relationship was observed between the HLA-DR types and caries incidence. In our study, there was no relationship between the dft-DMFT scores and HLA-DRB1 and HLA-DQB1 alleles.

Acton et al[12] have shown that DRB1*04 allele frequencies in African American women are positively associated with MS levels. They also observed a significant association between DRB1*3 and higher MS CFUs and between DRB1*8 and higher levels of MS as a percentage of total streptococci.[12] Ozawa et al[50] reported that salivary numbers of MS were weakly associated with only HLA-DQB1*0601.

Wallengren et al[16] showed lower salivary IgA activity to *S mutans* in HLA-DRB1*0401 and HLA-DRB1*0404. They also showed that HLA-DRB1*04 was not found in immuno-suppressed renal transplant individuals who had low levels of MS colony forming units (CFUs), but was present in 48% of those with high levels.[16] In healthy individuals, there was an insignificant increase in the frequency of HLA-DRB1*04 in those with high levels of MS. Among a group of dental faculty, students, and staff, the frequency of HLA-DRB1*04 had also increased in those with high levels of MS.[51] This difference, however, was not statistically significant. Finally, Wallengren et al[40,52] reported that the HLA DRB1*04 antigen correlated positively with MS colonization and may play a role in controlling dental caries. In our study, no association was observed between HLA-DRB1*04 and MS.

In the present study, the chi-square test showed that HLA-DRB1*01 and HLA-DQB1*03 were associated with the salivary numbers of MS. This association, however, was not confirmed when the data were subjected to logistic regression. Since the presence/absence of alleles is a binary variable for each individual, logistic regression had to be carried out—including other variables such as MS (high/low)—to predict the frequency of the alleles. Using a significance level of 5%, 1 in 20 tests will be significant by chance. Of the 38 different tests carried out (19 alleles for both DRB1* and DQB1*) one would expect 2 to be significant by chance. According to the chi-square statistics, there were only 2 statistically significant results, leading to the conclusion that very little weight can be placed on these. With

the number of patients ranging from 24 to as high as 102 or 186, previous studies have reported associations to varying degrees.[12,15,50]

The results obtained in the present study fail to establish a direct association between HLA antigens DRB1 and DQB1 and salivary numbers of MS in the selected child population. Several factors may account for these results and the results of previous reports. Antigens are specific for different species of MS, and perhaps the dental plaque comprised different species in previous studies. It is also conceivable that the pattern of HLA polymorphisms varies somewhat between racial and ethnic groups.[12] If the numbers of subjects are higher, there may be stronger associations between HLA alleles and oral micro-organisms. Finally, further subgroups (eg, HLA DRB1 0101, 0102, etc) may be associated with high-caries/caries-free subjects and dft-DMFT scores, which warrant further research.

Conclusions

Based on this study's results, the following conclusions can be made:

1. There was no significant difference between the frequency of the studied human leukocyte antigen alleles in high-caries/caries-negative subjects and dft-DMFT scores. Investigation of further subgroups, however, may reveal significant associations.

2. The MS levels were not significantly related to age and gender.

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