J Periodont Res 2003; 38; 508–517
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JOURNAL OF PERIODONTAL RESEARCH
ISSN 0022-3484

Are there HLA combinations typical supporting for or making resistant against aggressive and/or chronic periodontitis?

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Stein J, Reichert S, Gautsch A, Machulla HKG. Are there HLA combinations typical supporting for or making resistant against aggressive and/or chronic periodontitis? J Periodont Res 2003; 38; 508–517. © Blackwell Munksgaard, 2003

Objective and background: Human leukocyte antigens (HLA)/alleles have been considered as risk factors for periodontal disease. However, data from HLA associations is not consistent. Diversity of HLA antigen combinations and en bloc inherited HLA alleles (haplotypes), as known in systemic diseases, can be variable factors in disease association. Therefore, the aim of this study was to investigate the incidence of HLA homozygosities, heterozygosities and estimated haplotypes in German Caucasian groups with generalized aggressive ($N = 50$) and chronic ($N = 102$) periodontitis in comparison to control probands without periodontitis ($N = 102$).

Methods: HLA-A, -B, -Cw, -DRB1, -DRB3/4/5, -DQB1 typing was carried out using both serologic (microlymphocytotoxicity test) and genomic (PCR-SSP: PCR with sequence specific primers) techniques. Frequencies of all homozygosities, heterozygosities and haplotypes were determined in all patients and controls.

Results: In both patient groups, associations to HLA homozygosities and heterozygosities were found. Most striking was the significantly lower frequency of HLA-DRBblank* homozygosity (non-DRB3*/DRB4*/DRB5*) in chronic periodontitis ($p < 0.05$), whereas HLA-DRB1*15 : DRB5*(DR51) : DQB1*06 showed a slightly higher homozygosity rate in all patients. As the combination HLA-A*02,A*03 was significantly decreased in aggressive periodontitis ($p < 0.05$), HLA-A*01,A*03 heterozygosity was significantly lowered in chronic periodontitis ($p < 0.05$). Among others, the known positive associations for HLA-A*68/69 (A28) and HLA-DRB1*04 were confirmed by the haplotypes HLA-A*68/69 : Cw*07 : B*18 in aggressive periodontitis ($p < 0.05$) and HLA-Cw*08 : B*14 : DRB1*04 in chronic periodontitis ($p < 0.05$).

Conclusion: The present study elucidates the variety of HLA associations and therefore the difficulty to assign single HLA markers to periodontal disease. Susceptibility/resistance of both aggressive and chronic periodontitis may rather be influenced by particular HLA marker combinations. Associated HLA haplotypes may be of further importance for unknown gene loci representing a part of the genetic background for periodontitis. The different associations in aggressive and chronic periodontitis indicate different susceptibility/resistance factors for both diseases.

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Key words: aggressive periodontitis; chronic periodontitis; haplotypes; heterozygosity; homozygosity; human leukocyte antigens

Accepted for publication March 3, 2003

There is growing evidence that genetic aspects play a role in the onset and severity of periodontal disease (1–6). Polymorphisms of the interleukin-1 gene cluster (2, 3), tumour necrosis factor α (TNF-α) (4, 5) and certain immunoglobulin receptors (Fcγ-R) (6) have become the subject of previous investigations. This aside, numerous studies have pointed to the contribution of the major histocompatibility complex (MHC) as a potential genetic factor in aetiopathogenesis of periodontal diseases. HLA (human type of MHC) antigens play a crucial role in the recognition of foreign antigens. Due to the extensive polymorphism of HLA gene products, interindividual differences in immune response against bacterial antigens can be assumed. Among other diseases, several studies report a correlation between certain HLA antigens and periodontitis (7–20).

In our previous study we reported that certain HLA markers were significantly positively (HLA-A*11, -A*29, -B*14, -Cw*08, -DRB1*13) and significantly negatively [HLA-A*03, -A*30, -A*30/31 and -DRBblank* (non-DRB3/4/5)] associated with chronic or aggressive periodontitis, respectively, (21). Although there were clear associations found, there is a lack of consensus to previously released reports on HLA and periodontitis. Ethnographic influences and methodical (study population, HLA typing methods) differences may only be a minor reason for this inconsistency. Since HLA antigens are inherited codominantly and the alleles of the HLA loci are in linkage disequilibrium with each other, the manner and strength of HLA association of single HLA markers must be dependent from certain combinations of the participating HLA antigens or haplotypes, respectively, representing an additional variable factor in terms of potential disease association. This aspect has not been considered sooner because most of the prior studies, including the authors' previous work (21), were based on phenotype frequencies of single HLA markers only.

Investigation of HLA antigen combinations is based on the following ideas.

(i) HLA homozygosities could point to recessive susceptibility/resistance genes in linkage with homozygous HLA antigens.
(ii) Synergistic or antagonistic effects of HLA antigens have already been demonstrated in several systemic diseases such as type 1 diabetes mellitus (22, 23) or rheumatoid arthritis (24).
(iii) As disease disposition could be caused by alleles of several genes that are in linkage with each other (25), association of HLA haplotypes have a particular meaning in HLA association studies. This has also been shown in different diseases (26–28).

However, none of these investigations have been done on patients with periodontal disease. For this reason and based on the existence of multiple alleles of the MHC, the aim of the study was to carry out combination analyses of HLA markers, investigate the presence of associated homo- and heterozygosities and identify estimated haplotypes in Caucasian patients with chronic and aggressive periodontitis as well as in periodontitis-free individuals of the same origin using both serologic and genomic HLA typing methods.

Material and methods

Study population

Fifty unrelated German Caucasian patients with generalized aggressive periodontitis and 102 unrelated German Caucasian patients with chronic periodontitis were compared with 102 unrelated German Caucasian individuals who were free from periodontitis up to 38 years of age. Furthermore, a group with 157 unrelated German Caucasian blood donors were included in the study, representing the distribution of HLA markers in our geographical region. In all patients and periodontitis-free control probands, clinical probing depth and clinical attachment level were measured on six sites of each tooth. Interproximal alveolar bone loss was assessed by panoramic and/or a series of single radiographs. Approximal plaque index (29) and modified sulcular bleeding index were determined in all cases (Table 1).

Diagnosis of aggressive periodontitis was established according to clinical criteria for generalized early-onset periodontitis described by Tonetti and Mombelli (30): onset of periodontitis under 35 years of age; at least eight teeth with an attachment loss of 4 mm or more, at least three affected teeth other than molars or incisors; vertical and horizontal bone loss in the affected sites detectable in the radiograph; bleeding on probing; minimal accumulation of mineralized plaque in

Table 1. Clinical parameters of all investigated cohorts

	Aggressive periodontitis	Chronic periodontitis	Non periodontitis controls	Blood donors
Number	50	102	102	157
Age (median)	33.0 (19–43)	52.5 (38–73)	61.0 (38–95)	29.2 (19–39)
Smoker (%)	35.6	44.0	32.2	–
Approximal plaque index (SD) (%)	42.7 (27.6)	18.8 (7.7)	56.9 (19.32)	–
Sulcular bleeding index (SD) (%)	55.4 (27.4)	19.1 (9.6)	60.8 (20.3)	–
Probing depth (SD) (mm)	5.7 (1.4)	4.9 (0.7)	1.9 (0.3)	–
Clinical attachment level (SD) (mm)	6.7 (1.6)	5.7 (1.2)	3.4 (1.2)	–

SD = standard deviation.

comparison to chronic periodontitis; increased mobility on certain teeth; rapid course. Diagnosis of chronic periodontitis was established by the following criteria: age of onset over 35 years; at least 10 teeth present; at least five teeth with an attachment loss of 4 mm or more; detectable alveolar bone loss in the radiograph; bleeding on probing; often high accumulation of mineralized plaque; increased mobility on certain teeth; slow course. The control probands without periodontitis were older than 38 years of age and did not present any pathological attachment loss (probing depth < 3.5 mm, no gingival recession due to periodontitis) and no alveolar bone loss in the radiographs. Intentionally, we selected controls who had inadequate oral hygiene with approximal plaque index $> 30\%$. As a random group representing the distribution of HLA alleles in the population, the blood donors were not examined concerning periodontal diseases. All patients and control individuals were free from general diseases that are known to be associated with HLA markers.

Serologic HLA typing

Anticoagulated blood samples (20 ml) were taken from all patients and controls. Lymphocytes as indicator cells were separated from peripheral blood by density gradient centrifugation (31). All probands were typed for HLA-A, -B, -Cw antigens (Lymphotype 144, Biotest, Dreieich, Germany) by standard NIH (National Institute of Health) microlymphocytotoxicity test following manufacturer's instructions.

Genomic HLA typing

In order to secure and extend the results of the serologic typing technique, additional genomic HLA typing was used. For this reason, DNA was prepared from blood leukocytes by salting out technique (32). All patients and control probands were DNA typed by standard PCR with sequence-specific primers (PCR-SSP) (Perkin Elmer, PE 9600, Weiterstadt, Germany) for HLA-A, -B, -Cw (Deutsche Dynal, Hamburg, Germany) and HLA-DRB1, -DRB3/4/5, -DQB1 markers in low-resolution technique (Histotype-DR, Histotype-DQ, BAG, Lich, Germany) according to the protocol provided by the manufacturer.

Quality control

HLA-typing quality was demonstrated by typing control samples from INSTAND (Institute for Standardisation and Demonstration in Medical Laboratories e.V., Düsseldorf, Germany) and International DNA Exchange, ULCA Tissue Typing DNA Laboratory (Los Angeles, USA).

Statistical analysis

HLA antigen frequencies were calculated by direct counting and given as percentage of the total number (N) of the members of one group. As HLA alleles are inherited codominantly, there were two HLA markers (one on each chromosome) to be found in each HLA gene locus being analysed. Due to serologic and low resolution genomic typing 20 HLA-A, 40 HLA-B, nine HLA-Cw, 13 HLA-DRB1 and seven HLA-DQB1 markers could be identified (Table 2). Homozygosity was assumed if only one HLA

Table 2. List of all investigated HLA markers

HLA-A	A*01, A*02, A*03, A*23 (9), A*24 (9), A*25 (10), A*26 (10), A*34 (10), A*66 (10), A*11, A*29 (19), A*30 (19), A*31 (19), A*32 (19), A*33 (19), A* 74 (19), A*68 (28), A*69 (28), A*36, A*80
HLA-B	B*51 (5), B*52 (5), B*07, B*08, B*44 (12), B*45 (12), B*13, B*14, B*15 (62), B*15 (63), B*15 (75), B*15 (76), B*15 (77), B*38 (16), B*39 (16), B*57 (17), B*58 (17), B*18, B*49 (21), B*50 (21), B*54 (22), B*55 (22), B*56 (22), B*27, B*35, B*37, B*40 (60), B*40 (61), B*41, B*42, B*46, B*47, B*48, B*53, B*59, B*67, B*15 (71), B*15 (72), B*73, B*78
HLA-Cw	Cw*01, Cw*02, Cw*03, Cw*04, Cw*05, Cw*06, Cw*07, Cw*08, Cw*blank
HLA-DRB1	DRB1*01, DRB1*15 (2), DRB1*16 (2), DRB1*03, DRB1*04, DRB1*11 (5), DRB1*12 (5), DRB1*13 (6), DRB1*14 (6), DRB1*07, DRB1*08, DRB1*09, DRB1*10
HLA-DR3/4/5	DRB3*52(DR52), DRB4*53(DR53), DRB5*51(DR51), DRBblank*
HLA-DQB1	DQB1*05 (1), DQB1*06 (1), DQB1*02, DQB1*0301/4 (3), DQB1*0302 (3), DQB1*0303 (3), DQB1*04

HLA-A, -B, -Cw were detected by microlymphocytotoxicity test and PCR-SSP. HLA-DRB1, -DRB3/4/5 and -DQB1 were detected only by PCR-SSP. The main markers are shown in parenthesis. Cwblank* = Cw*12–18. DRBblank* = absence of HLA supertypes DRB3/4/5.

Table 3. Number of all investigated HLA marker combinations

Combinations within one HLA locus	Number of investigated combinations	Combinations between two HLA loci	Number of investigated combinations
A, A	210	A, B	800
B, B	820	A, Cw	180
Cw, Cw	45	A, DRB1	260
DRB1, DRB1	91	A, DQB1	140
DRB3/4/5, DRB3/4/5	10	B, Cw	360
DQB1, DQB1	28	B, DRB1	520
		B, DQB1	280
		Cw, DRB1	117
		Cw, DQB1	63
		DRB1, DQB1	91

marker was detectable within one locus. The frequency of all heterozygosities within each HLA locus as well as all combinations between different HLA loci (two locus combinations) was determined in every possible constellation (Table 3). As family typing was not feasible, definite haplotypes could not be identified. However, combinations with a positive linkage disequilibrium, measured by delta value Δ (magnitude of linkage disequilibrium), have been regarded as estimated haplotypes according to the method described by Mattiuz *et al.* (33). The frequencies of HLA homozygosities, heterozygosities and estimated haplotypes were compared between patients (aggressive periodontitis, chronic periodontitis) and periodontitis-free controls. Statistical analysis was based on a 2×2 contingency table and chi-squared testing. *p*-values were corrected (p_c) by Yates's continuity correction ($p_c = p$ of chi-squared corrected) or Fisher's exact test if appropriate. The latter was used if less than five patients or probands were the carrier of a marker. Since no specific hypothesis was tested, the Bonferroni correction was applied by multiplying the *P*-values with the number of comparisons tested. A *P*-value of < 0.05 was considered statistically significant. The relative risk (RR) was determined by calculating the odds ratio of a two contingency table. A modification according to Haldane *et al.* (34) was used if there were less than five patients or probands tested positive for a HLA marker or combination. In case of an association, RR expresses the factor how much higher (RR > 1) or lower (RR < 1) the relative disease risk for the carrier is compared to the non-carrier.

Results

Clinical parameters

In a 4-year period we selected patients and controls which were treated in our Department of Operative Dentistry and Periodontology for HLA studies. In Table 1 the clinical findings and proportion of smokers in all cohorts are presented. Among aggressive periodontitis patients and controls, the percentage of smokers was similar, whereas chronic periodontitis patients showed a slightly higher proportion. As approximal plaque index and sulcular bleeding index for chronic periodontitis patients were recorded after initial therapy including scaling and root planing, this group showed the lowest values. Patients with aggressive periodontitis showed slightly higher values of probing depth and clinical attachment level than patients with chronic periodontitis; controls had the lowest scores.

Distribution of HLA homozygosities

Aggressive periodontitis group — Comparing the frequencies of HLA overall homozygosity rate (A, B, Cw, DRB1, DRB3/4/5/blank, DQB1), patients with aggressive periodontitis and controls did not show any significant differences. However, regarding the single HLA markers, patients showed slightly higher frequencies of HLA-DRB1*15, -DRB5*51(DR51) and -DQB1*06 homozygosity (Table 4).

Chronic periodontitis group — In the group of patients with chronic periodontitis there was a significantly ($p_c < 0.05$) lower frequency of homozygosity for HLA-DRBblank*, i.e. complete absence of the supertypes DRB3*(DR52), DRB4*(DR53) and DRB5*(DR51) as well as a (non-significantly) higher frequency of HLA-DRB5*51(DR51) homozygosity in comparison to the controls. All the other alleles within the HLA loci A, B, Cw, DRB1 and DQB1 did not show any deviations of homozygosity (Table 5).

Distribution of HLA heterozygosities

Aggressive periodontitis group — Regarding all deviations of HLA heterozygosities, a significantly higher frequency of the combination HLA-DQB1*06, DQB1*0303 as well as a significantly lower appearance of HLA-A*02, A*03 was determined among patients with aggressive periodontitis. Furthermore, HLA-DQB1*02, DQB1*0301/4 occurred significantly less frequently among individuals without periodontitis. All other HLA heterozygosities did not show any striking deviations (Table 6).

Table 4. Deviations of HLA homozygosities between patients with aggressive periodontitis and control probands

	AP ($N = 50$)		Controls ($N = 102$)			
HLA-	*n*	%	*n*	%	RR	p_c
DRB1*15	2 ↑	4.00	1	0.98	3.49	> 0.05
DRB5*51(DR51)	2 ↑	4.00	1	0.98	3.49	> 0.05
DRBblank*	1	2.00	5 ↑	4.90	0.54	> 0.05
DQB1*06	6 ↑	12.00	5	4.90	2.65	> 0.05

The arrows show the deviation of the frequencies within one group from the population of blood donors. AP, aggressive periodontitis; RR, relative risk; p_c, *p* corrected according to Fisher or Yates.

Table 5. Deviations of HLA homozygosities between patients with chronic periodontitis and control probands

	CP ($N = 102$)		Controls ($N = 102$)			
HLA-	*n*	%	*n*	%	RR	p_c
DRB1*15	4 ↑	3.92	1	0.98	3.09	> 0.05
DRB5*51(DR51)	5 ↑	4.90	1	0.98	3.82	> 0.05
DRBblank*	0	0.00	5 ↑	4.90	0.09	0.035
DQB1*06	10 ↑	9.80	5	4.90	2.11	> 0.05

The arrows show the deviation of the frequencies within one group from the population of blood donors. CP, chronic periodontitis; RR, relative risk; p_c, *p* corrected according to Fisher or Yates.

Table 6. Deviations of HLA heterozygosities between patients with aggressive periodontitis and control probands

	AP ($N = 50$)		Controls ($N = 102$)			
HLA-	*n*	%	*n*	%	RR	p_c
A*02, A*03	1	2.00 ↓	12	11.76 ↑	0.22	0.040
DQB1*02, DQB1*0301/4	6	12.00	2	1.96 ↓	5.87	0.016
DQB1*06, DQB1*0303	5	10.00 ↑	2	1.96	4.86	0.039

The arrows show the deviation of the frequencies within one group from the population of blood donors. AP, aggressive periodontitis; RR, relative risk; p_c, *p* corrected according to Fisher or Yates.

Table 7. Deviation of HLA heterozygosities between patients with chronic periodontitis and control probands

	CP ($N = 102$)		Controls ($N = 102$)			
HLA-	*n*	%	*n*	%	RR	p_c
A*01, A*03	1	0.98 ↓	7	6.86 ↑	0.19	0.032

The arrows show the deviation of the frequencies within one group from the population of blood donors. CP, chronic periodontitis; RR, relative risk; p_c, *p* corrected according to Fisher or Yates.

Table 8. Deviations of two-locus combinations between in patients with aggressive periodontitis and control probands

	AP ($N = 50$)			Controls ($N = 102$)				
HLA-	*n*	%	Δ	*n*	%	Δ	RR	p_c
A*68/69 : B*18	3	6.00 ↑	0.018	0	0.00 ↓	−0.004	15.11	0.034
A*68/69 : Cw*07	7	14.00 ↑	0.007	4	3.92 ↓	−0.025	3.77	0.031
DRB1*04 : DQB1*0302	4	8.00 ↓	0.038	20	19.61 ↑	0.000	0.39	0.050
DRB1*13 : DQB1*06	17	34.00 ↑	0.124	19	18.63 ↓	−0.003	2.25	0.041

The arrows show the deviation of the frequencies within one group from the population of blood donors. Δ, linkage disequilibrium between the two HLA loci; AP, aggressive periodontitis; RR, relative risk; p_c, *p* corrected according to Fisher or Yates.

Table 9. Deviations of two-locus combinations of HLA markers between patients with chronic periodontitis and control probands

	CP ($N = 102$)			Controls ($N = 102$)				
HLA-	*n*	%	Δ	*n*	%	Δ	RR	p_c
A*11 : B*18	5	4.90 ↑	0.018	0	0.00	−0.002	11.56	0.030
Cw*08 : B*14	6	5.88	0.029	0	0.00 ↓	0.000	13.81	0.014
Cw*08 : DQB1*0302	4	3.92 ↑	0.015	0	0.00	0.000	9.37	> 0.05
B*14 : DRB1*04	4	3.92 ↑	0.014	0	0.00	0.000	9.37	> 0.05
B*14 : DQB1*0302	4	3.92 ↑	0.015	0	0.00	0.000	9.37	> 0.05
B*44 : DRB1*13	9	8.82 ↑	0.023	2	1.96	−0.018	4.08	0.029

The arrows show the deviation of the frequencies within one group from the population of blood donors. Δ, linkage disequilibrium between the two HLA loci; CP, chronic periodontitis; RR, relative risk; p_c, *p* corrected according to Fisher or Yates.

Chronic periodontitis group — Comparing the patients with chronic periodontitis and control probands, a significantly decreased frequency of the combination HLA-A*01, A*03 was found. No further deviations of HLA heterozygosities were discovered in this group (Table 7).

Two locus combinations and estimated haplotypes

Aggressive periodontitis group — The analysis of HLA marker combinations between different loci revealed a predominant appearance of HLA-A*68/69(A28) in the combinations HLA-A*68/69 : Cw*07 and -A*68/69 : Cw*07 which were significantly increased among patients with aggressive periodontitis. Moreover, in the patient group, HLA-DRB1*13 was expressed significantly more frequently in combination with HLA-DQB1*06 than with other HLA-DQB1 markers. HLA-DRB1*04 : DQB1*0302 appeared significantly less frequent among patients (Table 8). Due to the positive linkage disequilibrium of these combinations among the patients ($\Delta > 0$) estimated haplotypes could be defined. Figure 1(a) shows two haplotypes with significantly positive and one haplotype with a significantly lower frequency in aggressive periodontitis compared to the controls. All other estimated haplotypes in aggressive periodontitis did not show any deviations.

Chronic periodontitis group — In patients with chronic periodontitis, two locus combination analyses revealed HLA-B*14 to be significantly decreased in combination with HLA-Cw*08 ($p_c < 0.05$) among periodontitis-free controls and significantly increased in combination with HLA-DRB1*04 and HLA-DQB1*0302 among patients ($p_{uncorrected} < 0.05$, $p_c > 0.05$). The combinations HLA-A*11 : B*18 and -B*44 : DRB1*13 were statistically more frequent in the patient group (Table 9). Out of these combinations with positive linkage disequilibrium among the patients ($\Delta > 0$) three estimated haplotypes were determined, which were found significantly more frequently in patients with chronic periodontitis than in the controls (Fig. 1b). There were no more estimated haplotypes in chronic periodontitis with striking deviations.

Discussion

In the present study for the first time heterozygosity, homozygosity and

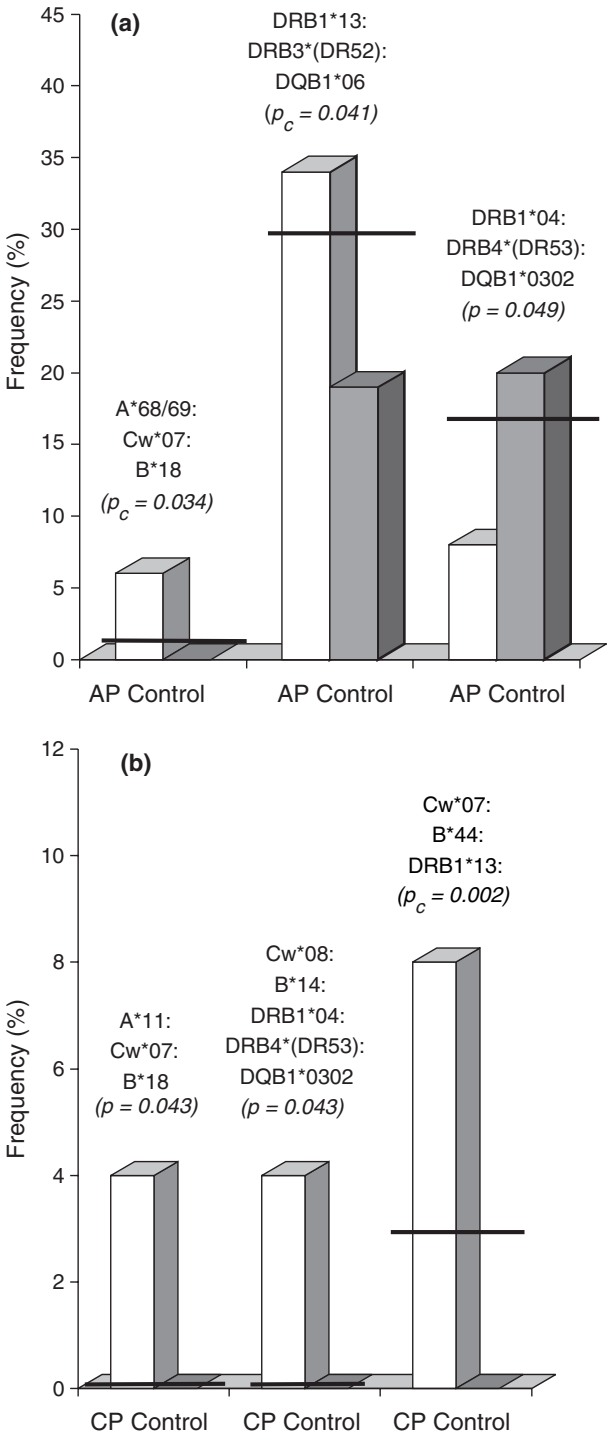

Fig. 1. Distribution of estimated HLA haplotypes among patients with aggressive (a) and chronic (b) periodontitis in comparison to control probands without periodontitis. The frequency of HLA haplotypes within the group of blood donors is marked with a crossbar. $p_c = p$ corrected according to Fisher or Yates; $p = p$ according to chi-squared test (uncorrected).

linkage disequilibrium of HLA markers were investigated in patients with aggressive periodontitis and chronic periodontitis. Contrary to most of the previous studies, the patients were compared with a selective group of probands which were free from periodontitis in order to provide a higher contrast between patients and controls. As a major risk factor, the smoking status was considered in patients and controls (Table 1). Although there were slightly more smokers among chronic periodontitis patients than in the younger group of aggressive periodontitis patients, the latter had higher values of probing depth and clinical attachment level. Obviously, smoking did not predominantly influence probing depth and clinical attachment level in comparison to other factors which are characteristic for both diseases. Blood donors were not used for direct comparison with the patient groups as they represent an inhomogeneous group of both individuals with several forms of periodontitis and healthy individuals. Therefore, they were involved to estimate the tendency of the HLA deviations between the patient groups and the control probands.

Patients with aggressive periodontitis and chronic periodontitis both revealed similar, but non-significant deviations of homozygosity rates of HLA-DRB1*15, -DRB5*51(DR51) and -DQB1*06 (Tables 4 and 5). The simultaneous increase of these markers can be explained by their strong linkage disequilibrium (35). The part of HLA-DQB1*0602 (high-resolution allele of HLA-DRB1*06) and DRB1*15 has already been discussed in Japanese patients with early-onset periodontitis. In those patients an atypical *Bam*HI restriction site in the HLA-DQB1 gene region pointed to a primary influence of the HLA-DQB1 locus (17), whereas Takashiba *et al.* (20) suggested HLA-DRB1*1501 to be the primary target/receptor/goal for antigenic peptides derived from Ag53 outer membrane protein of *Porphyromonas gingivalis*. Despite the missing significance and small number of probands, our results could be a hint for a recessive susceptibility due to HLA-DRB1*15 : DQB1*06 homozygosity. Compared to patients with chronic periodontitis, the significantly increased frequency of homozygosity for HLA-DRBblank*, i.e. absence of the supertypes HLA-DRB3*(DR52), DRB4*(DR53) and DRB5*(DR51), in the control group suggests that probands who do not carry any of the

markers HLA-DRB3*/4*/5* show higher resistance against periodontitis. Though significance of this result was lost according to the Bonferroni correction, the potential role of HLA-DRBblank* as a periodontitis resistance factor (RR = 0.09) is supported by two observations: HLA-DRBblank* was decreased in patients with aggressive periodontitis, which has already been published (21), and in all patients DRBblank* homozygosity was lowered (aggressive periodontitis) or missing (chronic periodontitis) compared with healthy controls. As immune-stimulating T cells can be activated by HLA class II antigens (36), the supertypes DRB3*/4*/5* might act as receptor molecules for pathogens which are presented to T-cell receptors of $CD4^+$ T helper cells. Apparently, in the absence of all HLA supertypes (homozygosity of DRBblank*) no pathogens would be available that could lead to increased periodontitis susceptibility ('low responder'). HLA-DRB5*51(DR51) or -DQB1*06 (linkage disequilibrium), however, might be better capable in binding antigen peptides than other HLA markers, leading to a higher responsiveness in homozygosity ('high responder').

For the calculation of HLA heterozygosities and two locus combinations an enormous number of comparisons were performed (Table 3). Although there were differences with statistical significance ($p < 0.05$) in the conservative sense (Tables 6–9), the results lost their significance when the Bonferroni correction was applied. Consequently, no strong association can be concluded. This is why the results have to be interpreted as tendencies towards an increase or decrease of certain combinations. Nonetheless, there have been remarkable deviations. HLA-A*03, which has been described to be more frequent among controls (21), showed different associations depending on the presence of HLA-A*02 or -A*01. Co-expression of HLA-A*01 may contribute to higher protection especially towards chronic periodontitis, and HLA-A*02 might increase resistance against aggressive periodontitis. Since a protective role of HLA-A2 has already been reported especially for juvenile (7, 8, 16) and rapidly progressive (16) periodontitis, our results could be an indication for a synergistic influence of HLA-A*03 in aggressive periodontitis. As HLA class I antigens present intracellular (self, viral) proteins, a relation to bacterial antigens does not appear conceivable. However, cross-reactions between bacterial antigens and HLA class I markers (molecular mimicry) have been described (37, 38). Furthermore, since virus infections have been reported to impair antibacterial defence in periodontitis (39), HLA class I dependent deficiencies of recognizing and binding viral antigen epitopes appear conceivable.

Regarding combination analysis between different HLA loci, three significantly associated estimated haplotypes were detected in aggressive periodontitis and in chronic periodontitis patients (Fig. 1). Estimating these haplotypes was possible because of the positive linkage disequilibrium of the associated HLA marker combinations. The following findings were striking. Firstly, depending on whether HLA-A*69/69 or -A*11 was part of the haplotype HLA-Cw*08 : B*18, an association to aggressive (HLA-A*68/*69) or chronic (HLA-A*11) periodontitis has been determined (Fig. 1). Together with the data of Reinholdt *et al.* (9), who found HLA-A28 (A*68/*69) to be increased in patients with juvenile periodontitis, a particular role of HLA-A28 for aggressive periodontitis can be assumed. HLA-A*11 has not been discovered by other authors before. Secondly, the increased frequency of the haplotype HLA-DRB1*13 : DRB3*(DR52) : DQB1*06 in the aggressive periodontitis group could be another hint for the role of HLA-DQB1*06 and also for HLA-DRB1*13, which was also increased as a single marker (21). This is plausible since in a parallel study, possible antigen binding motifs to *Porphyromonas gingivalis* have been described for anchor amino acid residues of the HLA-DQB1*0602 molecule as well as the HLA-DRB1*1301 and -DRB1*1302 molecules (40). In contrast to the results of Katz *et al.* (12), Firatli *et al.* (16) and Bonfil *et al.* (19) in patients with rapidly progressive periodontitis and juvenile periodontitis, no increased frequency of HLA-DR4 could be found in our patients with aggressive periodontitis, neither in phenotypes (21) nor in haplotypes or combinations. In chronic periodontitis patients, however, HLA-DRB1*04 was increased in unusual combination with the ancestral haplotype HLA-Cw*08 : B*14. This was unknown, since in prior studies only the phenotype frequency of the single marker HLA-DRB1*04 was associated with (chronic) adult periodontitis (14, 18).

The results presented in this study make it possible to differentiate unreported synergistic (e.g. HLA-A*01, A*03 heterozygosity in chronic periodontitis; HLA-A*01, A*02 heterozygosity in aggressive periodontitis; HLA-DQB1*06 homozygosity in aggressive and chronic periodontitis) and antagonistic (all those HLA antigens which were striking as single markers in previous studies but not present in combination analyses) effects possibly resulting from similar or different features of the HLA peptide binding paratopes. Two main conclusions are to be drawn.

(i) Both DRB1*15 : DQB1*06 homozygosity and DRB1*13 : DQB1*06 point to the potentially primary role of HLA-DQB1*06 as a risk factor for aggressive periodontitis.
(ii) The absence of all HLA-DR supertypes might be protective towards periodontitis.

Moreover, in the herein found estimated haplotypes an association seems to be referred to only one marker whereas the other HLA alleles are associated due to their linkage disequilibrium (e.g. HLA-A*68/69 in HLA-A*68/69 : Cw*07 : B*18 in aggressive periodontitis or HLA-A*11 in HLA-A*11 : Cw*07 : B*18 in chronic periodontitis) or another third unknown susceptibility gene associated with the haplotype. The different associations of the single HLA markers and/or some of the presented haplotypes suggest different susceptibility genes for aggressive periodontitis and chronic periodontitis, which could be useful for differential

diagnosis of aggressive and chronic periodontitis and a more individualized therapy as a consequence. However, it should be emphasized that to date no particular HLA marker or haplotype can be assigned to a strong correlation with a certain periodontal disease. This can be seen by the fact that in all previous studies, including the present investigation, all results remained insignificant after using the Bonferroni correction. Table 10 gives a summary on the concordances and differences of all HLA associations found in our cohorts, including the results of our previous report [HLA phenotypes (21), homozygosities, heterozygosities and haplotypes] and the results of HLA

Table 10. Associated HLA phenotypes [Machulla et al. (21)], homozygosities, heterozygosities and estimated haplotypes (present study) in German patients with aggressive and chronic periodontitis in comparison to previously described HLA associations

Associated HLA markers in German patients with AP and CP		Associated HLA markers described in previous studies		
HLA-	Disease	HLA-	Disease	Authors
I. Concordance with other studies				
↓ A*02	AP	↓ A2	JP + Adult P	Terasaki *et al*. 1975 (7)
↓ **A*02,A*03** *heterozygosity*		↓ A2	JP + Adult P	Kaslick *et al*. 1980 (8)
		↓ A2	JP + RPP	Firatli *et al*. 1996 (16)
↑ A*68/*69 (A28)	AP	↑ A28	JP	Reinholdt *et al*. 1977 (9)
↑ **A*68/*69 : Cw*07 : B*18**				
↓ B*51	AP	↓ B5 (B51 + 52)	Adult P	Goteiner & Goldman 1984 (10)
↑ DRB1*15 : DRB5*(DR51) : DQB1*06 *homozygosity*	AP + CP	↑ DR2 (DR15 + 16)	JP	Cogen *et al*. 1986 (11)
		↑ DR2 (DR15 + 16)	JP + RPP	Izumi *et al*. 1990 (13)
		↑ DRB1*1501 : DQB1*0602	EOP	Ohyama *et al*. 1996 (17) & Takashiba *et al*. 1999 (20)
↓ **DRB1*04 : DRB4*(DR53) : DQB1*0302**	AP	↓ DRB1*0405, *0401	EOP	Ohyama *et al*. 1996 (17)
↑ **Cw*08 : B*14 : DRB1*04**	CP	↑ DR4	Adult P	Alley *et al*. 1993 (14)
		↑ DR4	Adult P	Dyer *et al*. 1997 (18)
II. Discrepancies with other studies				
↑ A*68/*69 (A28)	AP	↓ A28	Adult P	Goteiner & Goldman 1984 (10)
↑ **A*68/*69 : Cw*07 : B*18**		↓ A68	JP	Moses *et al*. 1994 (15)
↑ DRB1*15 : DRB5*(DR51) : DQB1*06 *homozygosity*	AP + CP	↓ DR2 (DR15 + 16)	JP	Moses *et al*. 1994 (15)
↓ **DRB1*04 : DRB4*(DR53) : DQB1*0302**	AP	↑ DR4; DR53	RPP	Katz *et al*. 1987 (12)
		↑ DR4	JP + RPP	Izumi *et al*. 1990 (13)
		↑ DR4	JP + RPP	Firatli *et al*. 1996 (16)
		↑ DRB1*0401, *0404, *0405, *0408	RPP	Bonfil *et al*. 1999 (19)
III. Previously unknown HLA associations				
↓ **A*03**	CP			
↓ **A*01, A*03** *heterozygosity*	CP			
↑ **A*11**	CP			
↑ **A*11 : Cw*07 : B*18**				
↑ **A*29**	AP + CP			
↓ **A*31**	AP			
↑ **Cw*08 : B*14**	CP			
↑ **DRB1*13**	AP			
↑ **DRB1*13 : DRB3*(DR52) : DQB1*06**				
↓ **DRBblank*** *homozygosity*	CP			
↑ **DQB1*02, DQB1*0301/4** *heterozygosity*	AP			
↑ **DQB1*06, DQB1*0303** *heterozygosity*	AP			

Significant deviations are printed in bold.
Adult P, adult periodontitis; AP, aggressive periodontitis; CP, chronic periodontitis; EOP, early-onset periodontitis; JP, juvenile periodontitis; RPP, rapidly progressive periodontitis; ↓, decreased frequency; ↑, increased frequency.

studies in other cohorts. The data illustrates the diversity of the associations and interactions. On the other hand, there are numerous potential T cell epitopes of periodontopathogenic bacteria and viruses which could act as MHC ligands (41). This is why, for clinical usage, the prognostic value of these markers has not become relevant until now. Nevertheless, some of the HLA antigens show similar associations in several studies. These antigens might modify genetic background but do not entirely determine disease susceptibility. Further more detailed investigations of HLA haplotypes, in particular on the presented HLA-DRB1 : DQB1 markers, in relation to potential bacterial or viral peptide binding motifs are necessary to verify the potential role of these HLA markers in periodontitis.

Acknowledgements

This work would have been impossible without the help of dentist E. Wilcken, MD. Furthermore, the authors would like to thank Mrs A. Schaaf for her excellent technical assistance.

References

1. Marazita ML, Burmeister JA, Gunsolley JC, Koertge TE, Lake K, Schenklein HA. Evidence for autosomal dominant inheritance and race – specific heterogenity in early onset periodontitis. *J Periodontol* 1994;**65:**623–630.
2. Kornman KS, Crane A, Wang HY *et al*. The interleucin-1 genotype as severity factor in adult periodontal disease. *J Clin Periodontol* 1997;**24:**72–77.
3. McDevitt MJ, Wang H-Y, Knobelman C, *et al*. Interleukin-1 genetic association with periodontitis in clinical practice. *J Periodontol* 2000;**71:**156–163.
4. Galbraith GM, Hendley TM, Sanders J, Palesch Y, Pandey JP. Polymorphic cytokine genotypes as markers of disease severity in adult periodontitis. *J Clin Periodontol* 1999;**26:**705–709.
5. Kinane DF, Hodge P, Eskdale J, Ellis R, Gallagher G. Analysis of genetic polymorphisms at the interleukin-10 and tumour necrosis factor loci in early-onset periodontitis. *J Periodont Res* 1999;**34:** 379–386.
6. Kobayashi T, van der Pol WL, van de Winkel JG, *et al*. Relevance of IgG receptor IIIb (CD16) polymorphism to handling of *Porphyromonas gingivalis*: implications for the pathogenesis of adult periodontitis. *J Periodont Res* 2000;**35:**65–73.
7. Terasaki PI, Kaslick RS, West TL, Chasens AI. Low HL-A2 frequency and periodontitis. *Tissue Antigens* 1975;**5:**286–288.
8. Kaslick RS, West TL, Chasens AI. Association between ABO blood groups, HL-A antigens and periodontal diseases in young adults. a follow-up study. *J Periodontol* 1980;**51:**339–342.
9. Reinholdt J, Bay I, Svejgaard A. Association between HLA-antigens and periodontal disease. *J Dent Res* 1977;**56:**1261–1263.
10. Goteiner D, Goldman MJ. Human lymphocyte antigen haplotype and resistance to periodontitis. *J Periodontol* 1984;**55:** 155–158.
11. Cogen RB, Roseman JM, Al-Joburi W, *et al*. Host factors in juvenile periodontitis. *J Dent Res* 1986;**65:**394–399.
12. Katz J, Goultschin J, Benoliel R, Brautbar C. Human leukocyte antigen (HLA) DR4. Positive association with rapidly progressing periodontitis. *J Periodontol* 1987; **58:**607–610.
13. Izumi Y, Nitta H, Ishikawa I, Baehni P. Association between HLA system and periodontal disease. *J Parodontol* 1990;**9:**145–149.
14. Alley CS, Reinhardt RA, Maze CA, *et al*. HLA-D and T lymphocyte reactivity to specific periodontal pathogens in type 1 diabetic periodontitis. *J Periodontol* 1993;**64:**974–979.
15. Moses JH, Tsichti P, Donaldson P, Smith PB, Johnson NW, Bodmer JG. HLA and susceptibility to juvenile periodontitis in Afro-Caribbeans. *Tissue Antigens* 1994;**43:**316–319.
16. Firatli E, Kantarci A, Cebeci I, *et al*. Association between HLA antigens and early onset periodontitis. *J Clin Periodontol* 1996;**23:**563–566.
17. Ohyama H, Takashiba S, Oyaizu K, *et al*. HLA Class II genotypes associated with early-onset periodontitis: DQB1 molecule primarily confers susceptibility to the disease. *J Periodontol* 1996;**67:**888–894.
18. Dyer JK, Peck MA, Reinhardt RA, *et al*. HLA-D types and serum IgG responses to Capnocytophaga in diabetes and periodontitis. *J Dent Res* 1997;**76:**1825–1832.
19. Bonfil JJ, Dillier FL, Mercier P, *et al*. A 'case control' study on the role of HLA DR4 in severe periodontitis and rapidly progressive periodontitis. *J Clin Periodontol* 1999;**26:**77–84.
20. Takashiba S, Ohyama H, Oyaizu K, Kogoe-Kato N, Murayama Y. HLA genetics for diagnosis to early-onset periodontitis. *J Periodont Res* 1999;**34:**374–378.
21. Machulla HKG, Stein J, Gautsch A, Langner J, Schaller H-G, Reichert S. HLA-A, B, Cw, DRB1, DRB3/4/5, DQB1 in German patients suffering from rapidly progressive periodontitis (RPP) and adult periodontitis (AP). *J Clin Periodontol* 2002;**7:**573–579.
22. She JX, Bui MM, Tian XH, *et al*. Additive susceptibility to insulin-dependent diabetes conferred by HLA-DQB1 and insulin genes. *Autoimmunity* 1994;**18:**195–203.
23. Israel S, Kwon OJ, Weintrob N, *et al*. HLA class II immunogenetics of IDDM in Yemenite Jews. *Hum Immunol* 1998; **59:**728–733.
24. Zanelli E, Huizinga TW, Guerne PA, *et al*. An extended HLA-DQ-DR haplotype rather than DRB1 alone contributes to RA predisposition. *Immunogenetics* 1998; **48:**394–401.
25. Bodmer JG, Kennedy LJ, Lindsay L, Wasik AM. Applications of serology and the ethnic distribution of three locus HLA haplotypes. *Br Med Bull* 1987; **43:**94–121.
26. Sheehy MJ, Scharf SJ, Rowe JR *et al*. A diabetes-susceptible HLA haplotype is best defined by a combination of HLA-DR and -DQ alleles. *J Clin Invest* 1989; **83:**830–835.
27. Avila-Portillo LM, Vargas-Alarc'on G, Andrade F, Alarc'on-Segovia D, Granados J. Linkage disequilibrium of HLA-DR3 and HLA-DR4 with HLA-B alleles in Mexican patients with rheumatoid arthritis. *Clin Exp Rheumatol* 1994; **12:**497–502.
28. Price P, Calder DM, Witt CS *et al*. Periodontal attachment loss in HIV-infected patients is associated with the major histocompatibility complex 8.1 haplotype (HLA-A1,B8,DR3). *Tissue Antigens* 1999; **54:**391–399.
29. Lange DE, Plagmann HC, Eenboom A, Promesberger A. Clinical methods for the objective evaluation of oral hygiene. *Dtsch Zahnarztl Z* 1977;**32:**44–47.
30. Tonetti MS, Mombelli A. Early onset periodontitis. In: Lindhe J, Karring T, Lang NP, eds. *Clinical periodontology and implant dentistry*. Copenhagen: Munksgaard, 1998: 226–257.
31. Böyum A. A one-stage procedure for isolation of granulocytes and lymphocytes from human blood. *Scand J Clin Lab Invest* 1968;**21:**77–89.
32. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988;**16:**1215–1218.
33. Mattiuz PL, Ihde D, Piazza A, Ceppellini R, Bodmer WF. New approaches to the population genetic and segregation analysis of the HL-A system. In: Terasaki PI, ed. *Histocompatibility testing*. Copenhagen: Munksgaard, 1970: 193–205.
34. Haldane JBS. The estimation and significance of the logarithm of a ratio of

frequencies. *Ann Hum Genet* 1956;**20:** 309–311.

35. Gjertson DW, Lee S-H. HLA-A/B and -DRB1/DQB1 allele-level haplotype frequencies. In: Gjertson DW, Terasaki PI, eds. *HLA 1998*, Chapter 8. Lenexa/Kansas, USA: ASHI, 1998, 365–450.
36. Meuer SC, Schlossmann SF, Reinherz E. Clonal analysis of human cytotoxic T lymphocytes T4* and T8* effector T-cells recognize products of different major histocompatibility complex regions. *Proc Natl Acad Sci USA* 1982;**79:**4395.
37. Ebringer A. Ankylosing spondylarthritis, HLA B27 and the theory of crossed tolerance. *Rev Rhum Mal Osteoartic* 1983; **50:**763–769.
38. Hirata AA, Terasaki PI. Cross-reactions between streptococcal M proteins and human transplantation antigens. *Science* 1970;**168:**1095–1096.
39. Contreras A, Slots J. Herpesviruses in human periodontal disease. Short review. *J Periodont Res* 2000;**35:**3–16.
40. Reichert S, Stein J, Gautsch A, Langner J, Schaller HG, Machulla HKG. Gender differences in HLA phenotype frequencies found in German patients suffering from generalized aggressive periodontitis and chronic periodontitis. *Oral Microbiol Immunol* 2003;**17:**360–368.
41. Rammensee HG, Bachmann J, Emmerich NPN, Bachor OA, Stevanovic S. SYFPEITHI. database for MHC ligands and peptide motifs. *Immunogenetics* 1999; **50:**213–219.