Brazilian Journal of Medical and Biological Research (2008) 41: 493-499
ISSN 0100-879X

A majority of Brazilian patients with rheumatoid arthritis HLA-DRB1 alleles carry both the HLA-DRB1 shared epitope and anti-citrunillated peptide antibodies

P. Louzada-Júnior[1], M.V.C. Freitas[1], R.D.R. Oliveira[1], N.H.S. Deghaide[1], R.A. Conde[2], M.B. Bertolo[2] and E.A. Donadi[1]

[1]Divisão de Imunologia Clínica, Departamento de Clínica Médica, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brasil
[2]Disciplina de Reumatologia, Faculdade de Ciências Médicas, Universidade Estadual de Campinas, Campinas, SP, Brasil

Correspondence to: P. Louzada-Júnior, Divisão de Imunologia Clínica, Departamento de Clínica Médica, FMRP, USP, Av. Bandeirantes, 3900, 14048-900 Ribeirão Preto, SP, Brasil
Fax: +55-16-3633-6695. E-mail: plouzada@fmrp.usp.br

The objective of the present study was to evaluate the contribution of the shared epitope (SE), the rheumatoid arthritis (RA) protection model, and the occurrence of anti-cyclic citrullinated peptide (anti-CCP) antibodies in RA patients from a genetically diverse population. One hundred and forty Brazilian RA patients and 161 matched controls were typed for HLA-DRB1 alleles using amplified DNA hybridized with sequence-specific oligonucleotide probes or primers. Patients were stratified according to the presence or absence of SE (DRB1*0401, *0404, *0405, *0101, *1001, and *1402), of the DERAA alleles (DRB1*0103, *0402, *1102, *1103, *1301, *1302, and *1304), and X (all other alleles). Anti-CCP antibodies were measured by ELISA. The combined frequency of SE-positive alleles was significantly greater (76.4 *vs* 23.6%, $P < 0.0001$) than the controls. The SE/SE and SE/X genotypes were over-represented ($P < 0.0001$, OR = 6.02) and DERAA/X was under-represented in RA patients ($P < 0.001$, OR = 0.49), whereas the frequencies of the SE/DERAA, X/X and X/DERAA genotypes were not significantly different from controls. The frequency of anti-CCP antibodies was higher in SE-positive patients than in SE-negative patients (64.6 *vs* 44.7%, $P = 0.03$; OR = 2.25). Although the Brazilian population is highly miscegenated, the results of this study support the findings observed in most genetically homogeneous populations with RA; however, they are not mutually exclusive but rather complementary. The participation of DRB1-DERAA alleles in protection against RA was also observed (OR = 0.4; 95%CI = 0.23-0.68).

Key words: Susceptibility to rheumatoid arthritis; HLA; Brazilians; Shared epitope; Anti-cyclic citrullinated peptide

Research supported by CNPq, Fundação de Apoio ao Ensino, Pesquisa e Assistência do Hospital das Clínicas de Ribeirão Preto (FAEPA). Publication supported by FAPESP.

Received August 8, 2007. Accepted May 7, 2008

Introduction

Several lines of evidence indicate the role of major histocompatibility complex class II molecules in the susceptibility to seropositive rheumatoid arthritis (RA), particularly the molecules encoded by HLA-DRB1 genes (1). DRB1 genes that confer susceptibility to RA include DRB1*0401 and *0404 alleles among Caucasians (2,3), DRB1*0405 in East Asian patients (4), DRB1*0101 in Asian Indians (5) and Ashkenazi Jews (6), DRB1*1001 in Spaniards (7) and Blacks from South Africa (8), and DRB1*1402 in native North Americans (9). These RA-associated alleles share a nucleotide sequence encoding amino acid residues from position 67 to 74 of the third

hypervariable region (HV3) of the DRß1 chain, a sequence that has been named the RA shared epitope (SE) (10,11). The QRRAA sequence is found in molecules encoded by DRB1*0404, *0405, *0101, *1001, and *1402 alleles (sharing arginine at codon 71), and the QKRAA motif is observed in the DRB1*0401 allele (lysine at codon 71) (11).

In addition to the association of RA and SE-positive (SE^+) alleles, SE-negative (SE^-) alleles are also reported to influence susceptibility to RA by means of particular polymorphic anchor residue sequences present in one of the 5 peptide-binding pockets of the DRß1 chain, i.e., pockets 1, 4, 6, 7, and 9 (P1, P4, P6, P7, and P9). These pockets may influence the peptide-binding specificity of human leukocyte antigen (HLA) class II molecules, particularly P4 (12). The SE-associated alleles contribute only 3 residues (70, 71, and 74) to form P4, whereas the fourth residue is encoded in the first hypervariable region by codon 13. Alleles which have a neutral or negative electric charge in their P4, including DRB1*0103, *0402, *07, *08, *11 (except *1107), *12, and *13, are reported to protect against the development of RA. In contrast, alleles that possess a positive electric charge in their P4, such as DRB1*03, *0403, *0406, *0407, *0901, *1107, *14, *15, and *16 alleles, have no influence on predisposition to RA (12,13).

According to the RA protection (RAP) model, a short amino acid sequence, ^{70}DERAA^{74}, encoded by the HV3 of the DRB1*0103, *0402, *1102**,** *1103, *1301, 1302, and *1304 alleles confers protection against the development of RA. This model has been supported by several cross-sectional studies among Caucasian RA patients (14-16).

Autoantibodies to cyclic citrullinated peptides (anti-CCP antibodies) are highly specific for RA, can be detected years before the first clinical manifestation of RA (17), and are reported to be a good predictor of the development of RA (18). Because the contribution of the SE-containing HLA alleles to the pathogenesis of RA is not well understood, the novel information on the association of SE alleles with anti-CCP-positive disease (17) led us to evaluate the hypothesis that the SE alleles are a risk factor for anti-CCP antibodies.

Since the Brazilian population is genetically diverse, presenting a distinctive pattern of DR alleles and haplotypes, the evaluation of RA susceptibility models in this highly miscegenated population can contribute to the understanding of such associations. In the present study, we evaluated the contribution of DRB1 alleles stratified according to the clustering of SE^+ and SE^- alleles, the RAP model alleles and the presence of anti-CCP antibodies among Brazilian patients with RA.

Subjects and Methods

Patients and controls

A total of 140 (109 women) patients aged 24-79 years (median = 51), including 98 Whites, self-described to be of Western and Southern European ancestry, 24 Mulattoes (Caucasian and Black admixtures), and 18 Blacks, historically of mostly Bantu, Benin and Senegal African ancestry (19) presenting RA were evaluated at the University Hospital of the Faculty of Medicine of Ribeirão Preto, Ribeirão Preto, SP, Brazil, and at the University Hospital of the Faculty of Medical Sciences of Campinas, Campinas, SP, Brazil. All patients were typed for HLA-DRB1 alleles and fulfilled the 1987 revised criteria of the American College of Rheumatology for the diagnosis of RA (20). As controls, we evaluated 161 randomly selected normal blood donors of similar ethnic background from the same geographical region (21), including 86 Whites, self-described to be of Western and Southern European ancestry, 47 Blacks (Black ancestry up to the third generation), historically of mostly Bantu, Benin and Senegal African ancestry, as reviewed by Figueiredo et al. (19), and 28 Mulatto (Caucasian and Black admixtures) individuals. The population assignments were made by the same investigator who asked the donors about their ancestry. All individuals were submitted to a medical examination that showed no evidence of previous underlying diseases, and presented negative serology for Chagas' disease, B- and C-hepatitis, and HIV infection. The study protocol was approved by the Ethics Committee of the Faculty of Medicine of Ribeirão Preto, University of São Paulo, and all subjects gave written informed consent to participate in the study.

Autoantibody detection

The detection of anti-CCP2 IgG antibodies was performed using commercially available ELISA kits containing synthetic peptides (Quanta Lite anti-CCP2 Inova, San Diego, USA). ELISA was performed according to manufacturer instructions. Serum samples presenting results >20 U/mL were considered to be positive for the anti-CCP antibody. Rheumatoid factor was evaluated in all patients by nephelometry.

HLA-DRB1 typing

HLA-DRB1 alleles were characterized using polymerase chain reaction-amplified DNA hybridized to sequence-specific oligonucleotide probes, as previously described (22-24).

Allele clustering according to the SE and the RAP models

The frequency of DRB1 alleles considered to pertain to

the SE was as the sum of the individual frequency of the DRB1*0101, *0102, *0401, *0404, *0405, *0408, *1001 and *1402 alleles. Patients carrying one or two alleles of shared epitope were classified as SE-positive.

Protective DRB1 alleles (all carrying the HV3 motif DERAA) are the *0103, *0402, *1102, *1103, *1301 and *1302 alleles, which have been reported to be DERAA-positive alleles (12,13). For clarity, this study uses the term DERAA-encoding alleles but does not differentiate between the direct effect of these alleles and the effect of other alleles in linkage with the DERAA-encoding alleles. For analysis, 6 groups were formed according to the presence of DRB1 alleles, as follows: group A, homozygosity for the SE (SE/SE); group B, 1 SE allele and 1 no SE and no DERAA (SE/X); group C, 1 SE and 1 DERAA allele (SE/DERAA); group D, no SE and no DERAA (X/X); group E, 1 DERAA allele (X/DERAA), and group F, homozygosity for DERAA alleles (DERAA/DERAA) (see Table 2).

Statistical analysis

HLA frequencies observed in patients and controls were compared using the two-tailed Fisher exact test with the approximation of Woolf. Differences were considered to be significant at $P < 0.05$. The odds ratio (OR) was used to estimate the strength of the associations. The detection of the strongest association between alleles was performed by the method of Svejgaard and Ryder (25). The variables (gender, race, age) observed in patients and controls were compared using unpaired *t*-test. Differences were considered to be significant at $P < 0.05$.

Results

Frequencies of DRB1 and DRB1 alleles clustered as SE and DERAA alleles

The individual comparison of the frequency of the HLA alleles associated with RA, i.e., the HLA-DRB1*0401, *0404 (*0408), *0405, *0101, and *1001 alleles, observed in this series was significantly different from the respective frequency observed in controls (Table 1). Nevertheless, when the frequency of the SE alleles was considered, it was observed in 39.6% of RA patients and in only 12.5% of the controls (Pc < 0.0001; Table 1), with an OR of 6.02. The frequency of DERAA alleles in RA patients was decreased when compared with controls (11.42 *vs* 20.8%, P = 0.003, OR = 0.49), conferring protection against the development of RA (Table 1).

Table 1. DRB1 and shared epitope (SE) allele frequencies among patients with rheumatoid arthritis (RA) and controls.

DRB1	Controls (N = 312 alleles)	Rheumatoid arthritis (N = 280 alleles)	OR	95%CI
*0101	14 (4.35%)	34 (12.1%)*	2.94	1.54-5.60
*0102	9 (2.8%)	0 (0%)		
*0103	1 (0.3%)	0 (0%)		
*1501	29 (10.35%)	21 (10.1%)		
*1502	4 (1.25%)	2 (0.7%)		
*1601	9 (2.8%)	9 (3.2%)		
*1602	4 (1.25%)	4 (1.4%)		
*0301	35 (10.85%)	25 (8.9%)		
*0302	7 (2.15%)	2 (0.7%)		
*0401	6 (1.85%)	21 (7.5%)*	4.25	1.69-10.67
*0402	4 (1.25%)	4 (1.4%)		
*0403	3 (0.9%)	2 (0.7%)		
*0404	7 (4.3%)	21 (7.5%)*	6.40	2.17-18.90
*0405	4 (1.25%)	13 (4.7%)*	3.84	1.24-11.94
*0406	1 (0.3%)	0 (0%)		
*0407	7 (2.15%)	0 (0%)		
*0411	7 (2.15%)	2 (0.7%)		
*1101	19 (5.8%)	7 (2.5%)*	0.39	0.16-0.95
*1102	9 (2.8%)	5 (1.8%)		
*1103	4 (1.25%)	2 (0.7%)		
*1104	11 (3.4%)	8 (2.8%)		
*1105	2 (0.6%)	0 (0%)		
*1201	5 (1.55%)	5 (1.8%)		
*1202	1 (0.3%)	0 (0%)		
*1301	22 (6.85%)	12 (4.3%)		
*1302	21 (6.55%)	12 (4.3%)		
*1303	2 (0.6%)	0 (0%)		
*1304	1 (0.3%)	1 (0.35%)		
*1401	12 (3.7%)	5 (1.8%)		
*1402	3 (0.9%)	6 (2.15%)		
*07	27 (8.4%)	23 (6.45%)		
*0801	5 (1.55%)	5 (1.8%)		
*0802	3 (0.9%)	1 (0.35%)		
*0804	7 (2.15%)	2 (0.7%)		
*0807	5 (1.55%)	0 (0%)		
*09	7 (2.15%)	10 (3.55%)		
*10	5 (1.55%)	16 (5.7%)*	4.38	1.58-12.15
SE-positive	39 (12.5%)	111 (39.6%)*	6.02	3.95-9.17
DERAA-positive	65 (20.8%)	32 (11.42%)*	0.49	0.31-0.77

Data are reported as number with percent in parentheses unless otherwise stated. SE-positive alleles: DRB1*0101, *0102, *0401, *0404 (*0408), *0405, *1001 and *1402 alleles; DERAA-positive alleles: DRB1*0103, *0402, *1102, *1103, *1301 and *1302 alleles. OR = odds ratio; 95%CI = confidence interval at 95%. HLA frequencies observed in patients and controls were compared using the two-tailed Fisher exact test with the approximation of Woolf. Differences were considered to be significant at $P < 0.05$.

Table 2. DRB1 genotype frequencies according to the presence of predisposing alleles (SE alleles) and protection alleles in rheumatoid arthritis (RA) patients and controls.

Group	DRB1 genotype	RA (N = 140)	Controls (N = 161)
A	SE/SE	26 (18.6%)	6 (3.7%)
B	SE/X	57 (40.7%)	25 (15.5%)
C	SE/DERAA	10 (7.1%)	10 (6.2%)
D	X/X	30 (21.4%)	70 (43.5%)
E	X/DERAA	12 (8.6%)	45 (28.0%)
F	DERAA/DERAA	5 (3.6%)	5 (3.1%)

Data are reported as number of individuals with percent within parentheses. The shared epitope (SE) alleles are *0101, *0102, *0401, *0404, *0405, *0408, *1001 and *1402. The DERAA alleles are DRB1*0103, *0402, *1102, *1103, *1301, *1302 and *1304. X = represents all other DRB1 alleles. Groups were formed according to the presence of DRB1 alleles, as follows: group A, homozygosity for the SE (SE/SE); group B, 1 SE allele and 1 no SE and no DERAA (SE/X); group C, 1 SE and 1 DERAA allele (SE/DERAA); group D, no SE and no DERAA (X/X); group E, 1 DERAA allele (X/DERAA), and group F, homozygosity for DERAA alleles (DERAA/DERAA).

Table 3. Demographic data and laboratory features of the Brazilian rheumatoid arthritis patients in the present study (N = 140).

Variables	RA (N = 140)	Controls (N = 161)
Gender		
Female	109 (77.8%)	120 (74.5%)
Male	31 (22.2%)	41 (25.5%)
Race		
White	98 (70%)	86 (53.4%)
Mulatto	24 (17.1%)	28 (17.4%)
Black	18 (12.9%)	47 (29.2%)
Age (years, mean ± SD)	50.41 ± 11.28*	35.5 ± 8.4
median	50	34
minimum-maximum	24-78	22-45
Age at onset of disease (years, mean ± SD)	38.5 ± 11.2	NA
minimum-maximum	17-57	
Time of disease (years, mean ± SD)	9.49 ± 8.44	NA
median	10.5	
minimum-maximum	1-38	
Anti-CCP-positive	81 (58%)	ND
RF-positive	85 (60.7%)	ND
SE-positive	93 (66.4%)	39 (12.5%)

Data are reported as number of individuals with percent in parentheses unless otherwise stated. RA = rheumatoid arthritis; Anti-CCP = anti-cyclic citrullinated peptide antibodies; RF = rheumatoid factor; SE = shared epitope; NA = not applicable; ND = not determined. The variables (gender, race, age) observed in patients and controls were compared using unpaired *t*-test. Differences were considered to be significant at $P < 0.05$. SE-positive observed in patients and controls were compared using the two-tailed Fisher exact test with the approximation of Woolf. Differences were considered to be significant at $P < 0.05$ (*$P < 0.0001$).

DERAA-positive alleles (RAP model), and the influence of the SE

To study the effect of the presence of the DERAA motif on susceptibility to RA, patients and controls were divided into 6 groups according to their HLA-DRB1 genotypes (Table 2). The overall effect of DERAA on the development of RA was assessed by comparing the presence of the DERAA motif (groups C, E, and F) versus its absence (groups A, B, and D). The presence of DERAA was underrepresented in the RA patients (19.3%) versus controls (37.2%), conferring an OR of 0.40 ($P = 0.0006$; 95%CI = 0.23-0.68). The effect of the DERAA on the absence of the SE alleles was assessed by comparing group E (X/DERAA) plus group F (DERAA/DERAA) with group D (X/X). DERAA subjects had a reduced risk of developing RA (OR = 0.7), although the observed effect was not statistically significant ($P = 0.6$; 95%CI = 0.39-1.59).

Association between SE individuals and presence of anti-CCP antibodies

Anti-CCP antibody was present in 58% of the patients and rheumatoid factor was present in 60.7%. All patients with positive anti-CCP antibody also presented positive rheumatoid factor. Ninety-three patients carried at least one dose of shared epitope (Table 3). The frequency of anti-CCP antibodies was higher in SE-positive patients (64.5%) when compared with SE-negative patients (44.7%; $P = 0.03$, OR = 2.63; see Table 4). The distribution of anti-CCP antibodies was analyzed according to specific SE-related alleles; we did not observe a higher frequency of anti-CCP antibodies in RA patients carrying DRB1*0401, *0404, and *0101 compared with patients who do not carry these alleles.

Table 4. Association of HLA-DRB1 shared epitope (SE) alleles and anti-cyclic citrullinated peptide (anti-CCP) antibodies in rheumatoid arthritis (N = 140).

SE status	Anti-CCP-positive	Anti-CCP-negative	OR (95%CI)	P
SE-positive	60 (64.5%)	33 (35.5%)	2.25 (1.1-4.6)	0.03
SE-negative	21 (44.7%)	26 (55.3%)		

Data are reported as number of individuals with percent in parentheses unless otherwise stated. Presence or not of anti-CCP antibodies in SE-positive or SE-negative RA patients was compared using the two-tailed Fisher exact test with the approximation of Woolf. Differences were considered to be significant at $P < 0.05$.

Discussion

The association between RA and HLA-DR4 antigens was first reported in the 1970s using cellular techniques (1), and has recently been reexamined at the genomic DNA level (2,3). The susceptibility to RA has been reported to vary according to the ethnic background of the patients, supporting the "shared epitope" hypothesis (10,11). The extraordinary genetic diversity of HLA alleles and haplotypes seen in highly miscegenated populations may result from the aggregation of distinctive alleles from separate racial and ethnic groups. The gene bank of the modern Brazilian population represents the contribution of individuals of varied racial and ethnic groups. Historic immigrations from Europe have distributed various Caucasian populations, mainly Portuguese, Spanish, Italian and German, across Brazil. Brazilian Amerindian populations still exist as semi-isolated tribal groups, but their genes are also represented in modern urban populations. In a previous study, we observed that the frequencies of DRB1*15/16 (*02), *03, *12, *13, and *09 were similar among Whites, Blacks and Mulattoes. In contrast, the frequencies of DRB1*01, *04, *07, *08, *11, and *14 varied between the different groups. In particular, DRB1*01 genes were detected at approximately the same frequency in Whites and Mulattoes, but were greater in Blacks. DRB1*04 was similar in Whites and Mulattoes, but less in Blacks. Regarding the frequency of individual alleles, HLA-DRB1*0101 and *1001 were significantly greater in Blacks compared with Whites (Pc = 0.01 and Pc = 0.01, respectively). The polymorphism of DRB1*04 alleles seen in Brazilian Whites was greater than that observed in the Black population (21). Since the DRB1*04, *01 and *10 allelic frequencies are different for White and Black individuals, Pina et al. performed a study on RA patients using exclusively Afro-Brazilian descendants (Pina FP, Conde RC, Louzada-Júnior P, Donadi EA, Bertolo MB, unpublished data). They concluded that Afro-Brazilians with RA inherited a few HLA genes, which are characteristic of RA not only in African people, but also in Europeans and Asians who originated the Brazilian population. In Chile, where HLA-DR4 encoding DRB1 alleles do not encode susceptibility to RA (26), HLA-DR9 was associated with RA (27). In Japan, DRB1*0901 was associated with RA after exclusion of alleles *0101 and *0405 (28). In Kuwait, the association of HLA-DR3 with RA is explained by a high frequency of HLA-DR3 together with a relatively low frequency of HLA-DR4 alleles (29). In the present study, we did not observe an association with either DRB1-09 or DRB1-03 alleles. In fact, all DRB1 SE alleles were observed in Brazilian RA patients, including the rare DRB1*1402 allele that is commonly observed in individuals of Amerindian heritage, reflecting the high diversity of the population of the present study (21). Thus, in highly miscegenated populations the evaluation of SE frequency instead of individual alleles pertaining to the SE may be more important in terms of immunogenetic susceptibility to RA.

The events involved in the pathogenesis of RA are still unclear, but certainly the etiology is multifactorial. The shared epitope of HLA-DRB1 is the most important genetic risk factor. Environmental risk factors are less understood. Smoking is a candidate, associated with increased citrullinated cyclic peptide antibodies (30-32). In this study, we investigated the associations between HLA class II alleles and RA and the protective effects of the DERAA-encoding HLA-DRB1 alleles on RA susceptibility. The question of whether the effect of DERAA is truly protective or is merely the result of the absence of predisposing SE encoding HLA-DRB1 alleles is the subject of some controversy. In the present study, the comparison of subgroups (Table 2) allowed differentiation of the effects of protection and non-protection. In addition, in our population, we showed that DERAA-encoding HLA-DRB1 alleles independently reduce the risk of developing RA. These findings agree with similar results observed in more homogeneous groups such as Dutch (13) and Swiss (33) populations.

It has been demonstrated that peptides carrying the DERAA motif are naturally processed by human antigen-presenting cells, and it has been suggested that the protective effect of DERAA is mediated by a specific protective T cell response (34). Although our results show that the presence of a predisposing haplotype is not required to observe the protective effect associated with DERAA, it is possible that the DERAA sequence, particularly the DRB1*13 alleles, not only protects against RA but is also associated with a milder outcome in other diseases, such as reduced progression to active chronic hepatitis C and B (35). Theses findings are intriguing and indicate the importance of elucidating the biologic pathways underlying these associations, because they might unveil new insight into immune regulation in relation to the HLA system.

What does this tell us about the disease mechanism? Assuming differential risks for no susceptibility alleles, this hypothesis is able to explain data in the literature by referring to mutations in amino acid sequence at positions 67-74 in the HLA-DRB1 molecule. Thus, this version supports the notion that this sequence is central in conferring susceptibility to RA and does not indicate a role for another gene within the HLA complex. The fact that local amino acid substitutions induce differential risks suggests that the interaction of this region with presented peptides, superantigens or invariant chain may be important in the

pathogenesis of this disease (13,14).

We observed a higher frequency of anti-CCP antibodies in SE-positive patients (64.5%) compared with SE-negative patients (44.7%). In contrast, we did not observe a higher frequency of anti-CCP antibodies in RA patients that carried specific SE-related alleles, such as DRB1*0401, *0404, and *0101. Huizinga et al. (17) reported that the SE alleles were only associated with anti-CCP-positive RA and not with anti-CCP-negative disease, indicating that the SE alleles are not associated with RA as such, but rather with a distinct phenotype of the disease. Although no formal conclusions on causality can be drawn from this association study, these findings suggest that anti-CCP antibodies mediate the association between SE alleles and RA (36). It would be of interest to repeat the present study by following the development of anti-CCP antibodies and RA in healthy asymptomatic persons with and without SE alleles. Nevertheless, the present findings constitute an important refinement of the long-known association between HLA and RA by indicating that the SE alleles are not primarily associated with RA, but rather with anti-CCP antibody positivity.

Although SE represents the major contribution to RA susceptibility, other genes acting individually or in concert with SE may also be involved (37,38). In addition, environmental factors may further induce epigenetic alterations contributing to RA pathogenesis (39,40).

We have presented several lines of evidence indicating the involvement of DRB1 alleles in the susceptibility to RA. The RAP, SE and P4 polymorphism models are all associated with the HV3 region of the DRB1 gene and are useful to understand the immunogenetic susceptibility to RA. The findings of the present study support current RA models, and also support the idea that these models are not mutually exclusive but rather complementary as proposed by Zanelli et al. (16). Even though the Brazilian population is highly miscegenated, overall the present findings agree with others observed in genetically more homogeneous populations.

References

1. Stastny P. Mixed lymphocyte cultures in rheumatoid arthritis. *J Clin Invest* 1976; 57: 1148-1157.
2. Nepom GT, Seyfried CE, Holbeck SL, Wilske KR, Nepom BS. Identification of HLA-Dw14 genes in DR4+ rheumatoid arthritis. *Lancet* 1986; 2: 1002-1005.
3. Wordsworth BP, Lanchbury JS, Sakkas LI, Welsh KI, Panayi GS, Bell JI. HLA-DR4 subtype frequencies in rheumatoid arthritis indicate that DRB1 is the major susceptibility locus within the HLA class II region. *Proc Natl Acad Sci U S A* 1989; 86: 10049-10053.
4. Ohta N, Nishimura YK, Tanimoto K, Horiuchi Y, Abe C, Shiokawa Y, et al. Association between HLA and Japanese patients with rheumatoid arthritis. *Hum Immunol* 1982; 5: 123-132.
5. Nichol FE, Woodrow JC. HLA DR antigens in Indian patients with rheumatoid arthritis. *Lancet* 1981; 1: 220-221.
6. Schiff B, Mizrachi Y, Orgad S, Yaron M, Gazit E. Association of HLA-Aw31 and HLA-DR1 with adult rheumatoid arthritis. *Ann Rheum Dis* 1982; 41: 403-404.
7. Sanchez B, Moreno I, Magarino R, Garzon M, Gonzalez MF, Garcia A, et al. HLA-DRw10 confers the highest susceptibility to rheumatoid arthritis in a Spanish population. *Tissue Antigens* 1990; 36: 174-176.
8. Pile KD, Tikly M, Bell JI, Wordsworth BP. HLA-DR antigens and rheumatoid arthritis in black South Africans: a study of ethnic groups. *Tissue Antigens* 1992; 39: 138-140.
9. Willkens RF, Nepom GT, Marks CR, Nettles JW, Nepom BS. Association of HLA-Dw16 with rheumatoid arthritis in Yakima Indians. Further evidence for the “shared epitope” hypothesis. *Arthritis Rheum* 1991; 34: 43-47.
10. Gregersen PK, Silver J, Winchester RJ. The shared epitope hypothesis. An approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis. *Arthritis Rheum* 1987; 30: 1205-1213.
11. de Vries N, Tijssen H, van Riel PL, van de Putte LB. Reshaping the shared epitope hypothesis: HLA-associated risk for rheumatoid arthritis is encoded by amino acid substitutions at positions 67-74 of the HLA-DRB1 molecule. *Arthritis Rheum* 2002; 46: 921-928.
12. Seidl C, Korbitzer J, Badenhoop K, Seifried E, Hoelzer D, Zanelli E, et al. Protection against severe disease is conferred by DERAA-bearing HLA-DRB1 alleles among HLA-DQ3 and HLA-DQ5 positive rheumatoid arthritis patients. *Hum Immunol* 2001; 62: 523-529.
13. van der Helm-van Mil AH, Huizinga TW, Schreuder GM, Breedveld FC, de Vries RR, Toes RE. An independent role of protective HLA class II alleles in rheumatoid arthritis severity and susceptibility. *Arthritis Rheum* 2005; 52: 2637-2644.
14. Mattey DL, Dawes PT, Gonzalez-Gay MA, Garcia-Porrua C, Thomson W, Hajeer AH, et al. HLA-DRB1 alleles encoding an aspartic acid at position 70 protect against development of rheumatoid arthritis. *J Rheumatol* 2001; 28: 232-239.
15. Reviron D, Perdriger A, Toussirot E, Wendling D, Balandraud N, Guis S, et al. Influence of shared epitope-negative HLA-DRB1 alleles on genetic susceptibility to rheumatoid arthritis. *Arthritis Rheum* 2001; 44: 535-540.
16. Zanelli E, Breedveld FC, de Vries RR. HLA class II association with rheumatoid arthritis: facts and interpretations. *Hum Immunol* 2000; 61: 1254-1261.
17. Huizinga TW, Amos CI, van der Helm-van Mil AH, Chen W, van Gaalen FA, Jawaheer D, et al. Refining the complex rheumatoid arthritis phenotype based on specificity of the HLA-DRB1 shared epitope for antibodies to citrullinated proteins. *Arthritis Rheum* 2005; 52: 3433-3438.

18. Rantapaa-Dahlqvist S, de Jong BA, Berglin E, Hallmans G, Wadell G, Stenlund H, et al. Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis. *Arthritis Rheum* 2003; 48: 2741-2749.
19. Figueiredo MS, Silva MC, Guerreiro JF, Souza GP, Pires AC, Zago MA. The heterogeneity of the beta s cluster haplotypes in Brazil. *Gene Geogr* 1994; 8: 7-12.
20. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988; 31: 315-324.
21. Louzada-Junior P, Smith AG, Hansen JA, Donadi EA. HLA-DRB1 and -DQB1 alleles in the Brazilian population of the northeastern region of the State of São Paulo. *Tissue Antigens* 2001; 57: 158-162.
22. Mickelson E, Smith A, McKinney S, Anderson G, Hansen JA. A comparative study of HLA-DRB1 typing by standard serology and hybridization of non-radioactive sequence-specific oligonucleotide probes to PCR-amplified DNA. *Tissue Antigens* 1993; 41: 86-93.
23. Kimura A, Sasazuki T. Eleventh International Histocompatibility Workshop reference protocol for the HLA DNA-typing technique. In: Tsuji K, Aizawa M, Sasazuki T (Editors), *HLA 1991. Proceedings of the Eleventh International Histocompatibility Workshop Conference held in Yokohoma, Japan, November 6-13*. Oxford: Oxford Science Publications; 1992. p 397-419.
24. Olerup O, Zetterquist H. HLA-DR typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours: an alternative to serological DR typing in clinical practice including donor-recipient matching in cadaveric transplantation. *Tissue Antigens* 1992; 39: 225-235.
25. Svejgaard A, Ryder LP. HLA and disease associations: detecting the strongest association. *Tissue Antigens* 1994; 43: 18-27.
26. Massardo L, Jacobelli S, Rodriguez L, Rivero S, Gonzalez A, Marchetti R. Weak association between HLA-DR4 and rheumatoid arthritis in Chilean patients. *Ann Rheum Dis* 1990; 49: 290-292.
27. Massardo L, Aguirre V, Garcia ME, Cervila V, Nicovani S, Gonzalez A, et al. Clinical expression of rheumatoid arthritis in Chilean patients. *Semin Arthritis Rheum* 1995; 25: 203-213.
28. Wakitani S, Imoto K, Murata N, Toda Y, Ogawa R, Ochi T. The homozygote of HLA-DRB1*0901, not its heterozygote, is associated with rheumatoid arthritis in Japanese. *Scand J Rheumatol* 1998; 27: 381-382.
29. Sattar MA, al-Saffar M, Guindi RT, Sugathan TN, White AG, Behbehani K. Histocompatibility antigens (A, B, C, and DR) in Arabs with rheumatoid arthritis. *Dis Markers* 1990; 8: 11-15.
30. Padyukov L, Silva C, Stolt P, Alfredsson L, Klareskog L. A gene-environment interaction between smoking and shared epitope genes in HLA-DR provides a high risk of seropositive rheumatoid arthritis. *Arthritis Rheum* 2004; 50: 3085-3092.
31. Linn-Rasker SP, van der Helm-van Mil AH, van Gaalen FA, Kloppenburg M, de Vries RR, le Cessie S, et al. Smoking is a risk factor for anti-CCP antibodies only in rheumatoid arthritis patients who carry HLA-DRB1 shared epitope alleles. *Ann Rheum Dis* 2006; 65: 366-371.
32. Oliveira RD, Junta CM, Oliveira FR, Silva LM, Donadi EA, Louzada-Junior P. Share epitope, citrullinated cyclic peptide antibodies and smoking in Brazilian rheumatoid arthritis patients. *Clin Rev Allergy Immunol* 2008; 34: 32-35.
33. Zanelli E, Huizinga TW, Guerne PA, Vischer TL, Tiercy JM, Verduyn W, et al. An extended HLA-DQ-DR haplotype rather than DRB1 alone contributes to RA predisposition. *Immunogenetics* 1998; 48: 394-401.
34. Snijders A, Elferink DG, Geluk A, van Der Zanden AL, Vos K, Schreuder GM, et al. An HLA-DRB1-derived peptide associated with protection against rheumatoid arthritis is naturally processed by human APCs. *J Immunol* 2001; 166: 4987-4993.
35. Hohler T, Gerken G, Notghi A, Knolle P, Lubjuhn R, Taheri H, et al. MHC class II genes influence the susceptibility to chronic active hepatitis C. *J Hepatol* 1997; 27: 259-264.
36. van der Helm-van Mil AH, Verpoort KN, Breedveld FC, Huizinga TW, Toes RE, de Vries RR. The HLA-DRB1 shared epitope alleles are primarily a risk factor for anti-cyclic citrullinated peptide antibodies and are not an independent risk factor for development of rheumatoid arthritis. *Arthritis Rheum* 2006; 54: 1117-1121.
37. Verpoort KN, Cheung K, Ioan-Facsinay A, van der Helm-van Mil AH, de Vries-Bouwstra JK, Allaart CF, et al. Fine specificity of the anti-citrullinated protein antibody response is influenced by the shared epitope alleles. *Arthritis Rheum* 2007; 56: 3949-3952.
38. Yamada R, Yamamoto K. Mechanisms of disease: genetics of rheumatoid arthritis - ethnic differences in disease-associated genes. *Nat Clin Pract Rheumatol* 2007; 3: 644-650.
39. Gaston JS. Cytokines in arthritis - the 'big numbers' move centre stage. *Rheumatology* 2008; 47: 8-12.
40. Criswell LA, Saag KG, Mikuls TR, Cerhan JR, Merlino LA, Lum RF, et al. Smoking interacts with genetic risk factors in the development of rheumatoid arthritis among older Caucasian women. *Ann Rheum Dis* 2006; 65: 1163-1167.