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Investigation of the HLA Component Involved in Rheumatoid Arthritis (RA) by Using the Marker Association–Segregation χ^2 (MASC) Method: Rejection of the Unifying-Shared-Epitope Hypothesis

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Summary

In order to investigate the HLA component involved in rheumatoid arthritis (RA), we tested genetic models by the marker association–segregation χ^2 (MASC) method, using the HLA genotypic distribution observed in a sample of 97 RA patients. First we tested models assuming the involvement of a susceptibility gene linked to the DR locus. We showed that the present data are compatible with a simple model assuming the effect of a recessive allele of a biallelic locus linked to the DR locus and without any assumption of synergistic effect. Then we considered models assuming the direct involvement of the DR allele products, and we tested the unifying-shared-epitope hypothesis, which has been proposed. Under this hypothesis the DR alleles are assumed to be directly involved in the susceptibility to the disease because of the presence of similar or identical amino acid sequences in position 70–74 of the third hypervariable region of the DRBI molecules, shared by the RA-associated DR alleles DR4Dw4, DR4Dw14, and DR1. This hypothesis was strongly rejected with the present data. In the case of the direct involvement of the DR alleles, hypotheses more complex than the unifying-shared-epitope hypothesis would have to be considered.

Introduction

The association between HLA class II genes and rheumatoid arthritis (RA) is now well established. Many population studies have shown that RA is associated with HLA-DR4 and HLA-DR1 (Winchester et al. 1977; Stastny 1978; Gregersen et al. 1987). In addition, family studies have shown a nonrandom familial segregation of RA with HLA (Khan and Khan 1986; Payami et al. 1986; Weeks and Lange 1988). These results support the hypothesis that factors located in the HLA region are involved in the genetic susceptibility to RA. However, the HLA component involved in RA is not yet clearly defined, and several hypotheses assuming the involvement either of the DR alleles themselves or of a linked gene have been proposed to account for this component (Maeda et al. 1981; Jaraquemada et al. 1986; Pile et al. 1992). One of these hypotheses posits the effect of a biallelic locus located in HLA, with one susceptibility allele in linkage disequilibrium with the HLA-DR4 and DR1 alleles. This hypothesis has been tested for different modes of inheritance, with various approaches (Thomson 1983; Payami et al. 1986; Rigby et al. 1991). While previous studies were inconclusive on the transmission mode, the study by Rigby et al. (1991), using the antigen-genotype-frequencies-among-patients (AGFAP) method, affected-parent-to-children transmission, and affected-sib-pair haplotype sharing, reached the conclusion that a recessive transmission

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was compatible with the observed data, while the additive and dominant transmission modes were rejected.

On the other hand, when the different DR4 specificities which correspond to the nucleotide polymorphism on the HLA-DRB1 gene have been studied, it has been shown in the caucasoid population that only the alleles Dw4 and Dw14 are associated with RA while Dw10 and Dw13 are not (Stastny 1978; Ohta et al. 1982; Nepom et al. 1986). Two recent works (Nelson et al. 1991; Wordsworth et al. 1992) have studied the HLA genotype distribution in RA patients, taking into account the DR4 specificities, to estimate the risk attributable to HLA genotypes. An increased risk for the DR4Dw4/DR4Dw14 heterozygotes was found in both studies, but mostly for severely affected RA patients in the study by Wordsworth et al. (1992). Those authors then proposed the involvement of synergistic mechanisms in RA. However, in those studies no test of models was performed to confirm this hypothesis.

Another hypothesis, particularly interesting from a pathophysiological point of view, is the hypothesis of shared epitopes involved in the susceptibility to RA (Gregersen et al. 1987; Wordsworth et al. 1989; Gao et al. 1990). This hypothesis was proposed after sequencing studies which allowed the identification of two similar amino acid sequences in position 70–74 of the third hypervariable region of the DRB1 gene–encoded molecules, shared by the RA-associated DR4 subtypes as well as by DR1 but not shared by non–RA-associated DR alleles. Involvement of a group of related epitopes in the susceptibility to RA is plausible at the functional level, first because of the localization of the epitopes in the third hypervariable region which constitutes in part the peptide-binding site (Gregersen et al. 1987), second because specific HLA-DR4Dw14 T cell clones could be stimulated by antigen-presenting cells from HLA DR4Dw14 as well as DR1 patients (Goronzy et al. 1986), and finally because a study in Yakima Indians showed a higher prevalence in RA patients, compared with controls, of HLA DR14Dw16, which, in position 70–74, shares identical amino acid sequence with the DR1 and the DR4Dw14 epitopes (Willkens et al. 1991). However, the hypothesis of the involvement of the HLA-DRB alleles in RA because of the presence of the shared epitopes has not yet been tested for fit to RA data.

The aim of our study was to test various genetic models proposed to explain the HLA component involved in RA. Models were tested with the marker association–segregation χ^2 (MASC) method (Clerget-Darpoux et al. 1988) by using the HLA genotypic distribution in a sample of 97 RA patients typed for the different HLA specificities.

Patients, Material, and Methods

Patients

Ninety-seven Caucasian patients suffering from classical RA (Arnett et al. 1988) were randomly selected from a pool of patients recruited from two centers (Service d'Immuno-Rhumatologie, CHRU Montpellier; and Service de Rhumatologie, CHRU Nîmes) to constitute the sample of RA patients. No selection criteria such as severity or extra-articular manifestations were used to select the patients. There were 79 females and 18 males, ranging in age from 26 to 84 years. The mean duration of the disease was 9.0 ± 0.8 years. Written informed consent was obtained from all the patients. The control group consisted of 1,302 randomly selected voluntary bone marrow donors from the Montpellier center.

Genomic DNA

Genomic DNA was extracted from individuals' peripheral blood cells by using either a treatment by proteinase K and a phenol/chloroform extraction (Grimberg et al. 1989) or salting-out procedure (Miller et al. 1989).

HLA-DR Oligotyping by Nonradioactive Reverse Dot-Blot Methodology

This methodology has been described elsewhere (Eliaou et al., in press). In brief, the nucleotide sequence of the primers used for the PCR amplification was chosen from the conserved regions of the second exon of the HLA-DRB1 gene. For the determination of the HLA-DR4 subspecificities, a sequence-specific primer was used instead of the "left" primer, under the same conditions, to generate DR4 group–specific amplifications. Two sets of sequence-specific oligonucleotides (SSOs) were used in this study, as described elsewhere (Eliaou et al., in press). First, a pannel of 15 SSOs was used to determine the patients' and controls' DRB1 generic specificities. Depending on the generic oligotyping, four SSOs were then designed to type for the main HLA-DR4 subspecificities—DR4Dw4 (DRB1*0401), DR4Dw10 (DRB1*0402), DR4Dw13 (DRB1*0403), and DR4Dw14 (DRB1*0404). The SSOs were chemically 3′-tailed with 25 thymidine residues during the automated synthesis on an Applied Biosystem 391 A DNA synthesizer (Applied Biosystems, Paris)

and then were blotted onto nitrocellulose membrane (Eliaou et al., in press).

Starting from 1 μg of genomic DNA, the HLA-DRB gene second exons were amplified and labeled by asymmetrical PCR, by using biotinylated 11-dUTP (Sigma Chimie, La Verpillière, France). Forty microliters of each individual asymmetrical PCR product were hybridized to a single nitrocellulose membrane blotted with tailed SSOs. After washing and saturation of the membranes, nonradioactive visualization of the reverse hybridization was performed by using 10 μg of streptavidin alkaline-phosphatase and its colorless chromogen substrates (BCIP and NBT) (Bethesda Research Laboratory, Cergy Pontoise, France), as recommended by the manufacturers.

Data Analysis

The MASC method.—MASC (Clerget-Darpoux et al. 1988) was designed for modeling and testing the role of a candidate gene. It uses two kinds of information on a marker of this candidate gene, one provided by linkage disequilibrium, if present, and one provided by the cosegregation of the marker and the disease. The principle of the MASC method is to categorize data into classes according to the different information available in the RA patients (index cases) and their relatives. A PASCAL program allows one to calculate the distribution expected in each category under a given genetic model and to compare this distribution with that observed by a χ^2 test. Minimization of the χ^2 allows both estimation of the parameters of the model and testing of whether the model fits the data. The number of df of the χ^2 is equal to the number of categories minus the number of estimated parameters. Because no information on the relatives of the index case was available, data were categorized for the HLA genotype of the index case only.

For categorization of the data in HLA genotypes, we considered the four DR alleles—DR4Dw4, DR4Dw14, DR1 (the RA-associated DR alleles), and DRX (all DR alleles are different from the previously given alleles). Thus we obtained 10 categories for the genotypes of the RA index case. Our aim was then to test the fit of genetic models to the distribution of these genotypes in RA patients.

Models.—The first model considered here is a model assuming the effect of one susceptibility allele (S1) of a biallelic (S1 and S2) locus located in HLA. Alleles at this locus are in linkage disequilibrium with HLA-DR alleles. The parameters of this model are F_{ij}, the penetrance of each genotype S_iS_j, and the coupling frequencies C_{ij}, the probability that an individual would carry the allele S_i, given that he carries the HLA-DR allele M_j. The frequencies of S1 and S2 can be deduced from the coupling frequencies and from the frequencies of the DR alleles. The DR allele frequencies have been estimated from the control group. Because HLA-DR4 subtype information was not available for this group, we used DR4 subtype distribution in the French population, obtained from data of the 11th HLA Workshop. This was appropriate because of similar frequencies of DR1 and DR4 observed in our control group and in the French population. Frequency estimates of DR4Dw4, DR4Dw14, DR1, and DRX are then equal, respectively, to .056, .03, .108, and .806. Better control frequencies would have been constituted by estimating DR allele frequencies on the basis of the nontransmitted parental alleles (Field 1989; Thomson et al. 1989). Unfortunately, this requires knowing the parental genotypes, which were unavailable in this study.

We tested the general biallelic-locus model (M1) including coupling frequencies for the four HLA-DR alleles and different submodels. Let M2 be the recessive model, and let M3 be the dominant one. In each submodel M4, M5, or M6, the coupling frequency for DR4Dw4, DR4Dw14, or DR1 is equal to that for DRX. Testing these models corresponds to testing absence of association of RA with either DR4Dw4, Dw14, or DR1. We then considered the model (M7) where the coupling frequencies for the three alleles HLA-DR4Dw4, Dw14, and DR1 are equal, to test whether the associations with the susceptibility allele S1 were equal for each RA-associated DR allele.

In addition, we proposed to test particular models which are of interest from a biological point of view. These models assumed the involvement, in the susceptibility to RA, of the epitopes shared by the alleles HLA DR4Dw4, Dw14, and DR1. These epitopes correspond to two amino acid sequences that are very similar but not identical. Two hypotheses could then be proposed —that the two epitopes either (1) have the same functional effect in the genetic susceptibility to RA or (2) do not. In the case of the first hypothesis, the alleles HLA DR4Dw4, Dw14, and DR1 correspond to a same susceptibility allele, its effect being due and only due to the presence of the shared epitopes. Note that this model (M8) is a particular submodel of M7, with coupling frequencies of the susceptibility allele with the three DR alleles equal to 1 and with DRX equal to 0.

We considered then the hypothesis of the involvement, in RA, of two epitopes with different effect, since the sequence shared by the RA-associated DR alleles is, in fact, strictly identical for DR4Dw14 and

Table 1

HLA Genotypic Distributions Observed in the RA Index Sample and Expected under the Tested Models

	Observed Distribution	Distribution Expected under Model								
		M1	M2	M3	M4	M5	M6	M7	M8	M9
DR4Dw14/DR4Dw1	3	3	3	1	0	2	2	1	1	1
DR4Dw14/DR4Dw4	7	4	5	2	1	2	4	3	3	3
DR4Dw14/DR1	4	7	8	5	1	5	3	5	5	5
DR4Dw14/DRX	19	19	17	28	4	26	19	8	8	9
DR4Dw4/DR4Dw4	0	2	2	1	0	0	2	2	2	0
DR4Dw4/DR1	6	5	6	3	2	2	2	9	9	11
DR4Dw4/DRX	12	13	12	14	19	4	16	16	16	10
DR1/DR1	3	4	5	3	1	3	1	9	9	8
DR1/DRX	26	22	20	25	17	25	10	30	30	34
DRX/DRX	17	17	20	15	54	28	39	14	14	15

DR1 (QKRAA in one-letter code) but differs, at one amino acid, from the sequence shared by DR4Dw4 (QRRAA). The model tested (M9) assumes that DR4Dw14 and DR1 correspond to one susceptibility allele and that DR4Dw4 corresponds to another one. The model M9 is then a particular submodel of the triallelic-locus model with two susceptibility alleles S1 and S2 and one nonsusceptibility allele S3. The parameters of this model are the penetrances F_{ij} and the coupling frequencies, with coupling frequencies of S1, S2, and S3 equal, respectively, to 1, 0, and 0 with DR4Dw14 and DR1; to 0, 1, and 0 with DR4Dw4; and to 0, 0, and 1 with DRX. The penetrance of the nonsusceptibility allele in homozygotes is not set to 0, in order to allow for presence of phenocopies or the effect of alternative risk factors.

Results

In table 1 is given the observed genotypic distribution in the sample of RA patients, as well as those expected under the models tested. In table 2 are indicated the parameter estimates and the χ^2 values obtained for the various models.

The general biallelic-locus model M1, assuming one susceptibility allele in linkage disequilibrium with DR alleles, is not rejected (χ^2=6.31; 5 df). The coupling frequency is the highest for DR4Dw14, smaller for DR4Dw4, and smallest for DR1. However, the difference in coupling frequencies between DR4Dw4 and DR1 is small. The estimated penetrance vector is not very far from that corresponding to recessive transmission: the recessive model M2 is indeed not rejected (χ^2=8.34; 5 df), while dominant transmission (model M3) is rejected (χ^2=19.47; 5 df). The observed proportions of DR4Dw14/DR4Dw4 and DR4Dw4/DR1 heterozygotes are higher, and those of DR4Dw14/DRX heterozygotes are smaller than the proportions expected under a dominant transmission, which leads to the rejection of this model while the observed distribution is compatible with recessive transmission.

Association of RA with DR4Dw4, DR4Dw14, and DR1 is confirmed by rejecting models M4, M5, and M6, corresponding, respectively, to absence of association with DR4Dw14 (χ^2=292; 6 df), DR4Dw4 (χ^2=49.86; 5 df), and DR1 (χ^2=56.55; 6 df). Equality of association between the susceptibility allele and the three DR alleles (M7) is also rejected (χ^2=38.55; 6 df). The proportion of DR4Dw14 either in homozygotes or in heterozygotes is too large, compared with those of DR4Dw4 and DR1, to be compatible with the hypothesis of equal association of RA with the three DR alleles. Thus the observed distribution can be well explained by the hypothesis of one susceptibility allele at a biallelic locus and in linkage disequilibrium with DR4Dw4, Dw14, and DR1.

Model M8, which assumes the involvement of the epitopes shared by DR4Dw4, DR4Dw14, and DR1, with an identical effect for each epitope, is rejected (χ^2 =38.55; 7 df). This result is not surprising, since this model is a particular submodel of M7, M7 itself being strongly rejected. The model M9 assuming the involvement of two epitopes, each having a different effect, one shared by DR4Dw14 and DR1 and the other specific to DR4Dw4, is also rejected (χ^2=31.39; 5 df). Note that, under this model, the penetrance estimate

Table 2

Parameter Values Which Minimize the χ^2 under the Tested Models

	Result under Model								
	M1	M2	M3	M4	M5	M6	M7	M8	M9
Penetrance:									
FS1S1	1[a]	1[a]	1[a]	.1[a]	1[a]	1[a]	1[a]	1[a]	.5[a]
FS1S2	.098	0	1[a]	.28	.23	0	.12	.22	.64
FS2S2	0	0	.001	0	0	0	0	.03	.0
FS1S3	...	...	...	...	...	...	...	...	.14
FS2S3	...	...	...	...	...	...	...	...	.08
FS3S3	...	...	...	...	...	...	...	...	.02
Coupling frequency:									
P(S1/DR4Dw14)	1	.82	.015	.20	.82	.91	.64	1[a]	1[a]
P(S1/DR4Dw4)	.35	.32	.004	1.	.04	.41	.64	1[a]	0[a]
P(S1/DR1)	.29	.27	.003	.29	.20	.14	.64	1[a]	1[a]
P(S1/DRX)	.04	.07	0	.20	.04	.14	.05	0[a]	0[a]
P(S2/DR4Dw14)	...	...	...	...	...	...	...	...	0[a]
P(S2/DR4Dw4)	...	...	...	...	...	...	...	...	1[a]
P(S2/DR1)	...	...	...	...	...	...	...	...	0[a]
P(S2/DRX)	...	...	...	...	...	...	...	...	0[a]
χ^2	6.31	8.34	19.47	291.7	49.86	56.55	38.55	38.55	31.39
df	4	5	5	6	5	6	6	7	4

NOTE.—There is sensibility for estimation only on the penetrance ratios (FS1S2/FS1S1. . .), but not on FS1S1, which was then set to 1 or, in the case of overdominance (FS1S2/FS1S1 >1), to a value <1.

[a] Fixed parameter.

for the homozygotes S2S2 converges to 0, which is due to the absence of DR4Dw4 homozygotes in our sample of RA patients. Nevertheless, this model is still rejected, because the associations of RA with DR4Dw14 and with DR1 are not equivalent, and this is not compatible with the hypothesis that DR4Dw14 and DR1 have the same effect.

Discussion

Regarding the HLA component involved in RA, we considered here two main hypotheses—one assuming the involvement of the DR alleles themselves and the other assuming the involvement of a linked locus. For the first case, it is proposed, as the unifying-shared-epitope hypothesis, that the direct involvement of the DR alleles is due to the presence of similar or identical amino acid sequences in position 70–74 of the third hypervariable region of the HLA-DRB1 molecule, shared by DR4Dw4, DR4Dw14, and DR1. We tested the model assuming that the effect of the DR alleles is due only to the presence of the related epitopes shared by these alleles, each epitope having the same effect. That corresponds to an identical involvement of the three DR alleles. When the HLA-DR genotypic distribution observed in the sample of RA patients was used, this hypothesis was strongly rejected in the present study. The observed difference in the level of association with RA, for the three alleles, indicates that, if these alleles are directly involved in RA, then they must not have the same effect. Although this result does not exclude the direct involvement of the DRB1 genes, it shows that their possible involvement cannot be explained by the presence of the shared epitopes only. Such a conclusion could also be drawn from the other previous studies, since the respective strengths of association of RA with the three alleles are always found to be very different. Note, however, that in caucasoid population studies heterogeneous results have been found for the gradient of strength of association of the DR alleles with RA, the strongest association being with DR4Dw4 in several studies (Gao et al. 1990; Nelson et al. 1991), while in the study of Ronningen et al. (1990) and in the present study the strongest association was found with DR4Dw14. In addition, no association with DR1 (Ronningen et al. 1990; Nelson et al. 1991) or DR4Dw14 (Zoschke and Segall 1986; Gao et al. 1990) was described in other studies. Nelson et al. (1991)

pointed out that these heterogeneous results, obtained particularly for DR4Dw14 and DR1, poorly supported the unifying-shared-epitope hypothesis.

In a second step, we tested the model assuming the involvement, in RA, of two epitopes with different effects, one epitope shared by DR4Dw14 and DR1 and the other shared by DR4Dw4. This model was also strongly rejected, because the difference in the level of association of RA with DR4Dw14 and DR1 is not compatible with the hypothesis of the same effect of both DR alleles. The hypothesis of the involvement of the three DR alleles, each with a different effect, cannot be tested with our data. Such a model, including the effect of three susceptibility alleles and one nonsusceptibility allele, would have a number of parameters which is higher than the number of categories studied. To test such models, additional information would have to be used, such as the parental disease status and the number of haplotypes shared by affected sibs.

Regarding the hypothesis of the role, in RA, of a locus linked to the DR locus, we found that a biallelic-locus model with one susceptibility allele in linkage disequilibrium with DR alleles is compatible with the observed HLA genotypic distribution in RA patients. In agreement with the study by Rigby et al. (1991), dominant transmission is strongly rejected while recessive transmission is compatible with the data. Note that an excess of DR4Dw4/DR4Dw14 heterozygotes compared with DR4Dw4 or DR4Dw14 homozygotes has been found in two studies (Nelson et al. 1991; Wordsworth et al. 1992). However, this excess was significant only in severely affected RA patients in the study by Wordsworth et al. (1992). Such results suggest the involvement of synergistic mechanisms in RA, perhaps more particularly in severe RA. In our study, RA patients were not selected for severity, and, although there is a slight excess of DR4Dw4/DR4Dw14 heterozygotes compared with DR4Dw4 homozygotes, the observed distribution is well explained by a biallelic-locus model, with one susceptibility allele in linkage disequilibrium with the DR alleles and without any assumption of synergic effect.

In conclusion, the RA-associated HLA-DR could be in linkage disequilibrium with a susceptibility allele, and we showed that a simple model of a recessive allele located at a locus linked to the DR locus explains well the present data. Another possibility is that RA-associated HLA-DR alleles could themselves be involved in RA. However, their involvement cannot be due solely to the presence of the amino acid sequences shared by these alleles. To test the direct involvement of the DR alleles, hypotheses more complex than the unifying-shared-epitope hypothesis have to be investigated.

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