Effect of Rheumatoid Arthritis (RA) Susceptibility Genes on the Immune Response to Viral Citrullinated Peptides in RA

To the Editor:

Anticitrullinated peptide/protein antibodies (ACPA) are a family of rheumatoid arthritis (RA)-specific antibodies that recognize various protein sequences containing citrulline, the deiminated form of arginine produced posttranslationally by peptidylarginine deiminase (PAD) enzymes. Recently, viral citrullinated peptides (VCP) derived from Epstein-Barr virus nuclear proteins EBNA-1 and EBNA-2 have been described as a target of ACPA in RA sera[1,2].

Genetic factors predisposing to RA, namely HLA-DRB1 shared epitope (SE) alleles and PTPN22 gene variants, as well as the TRAF1/C5 locus and PADI4 susceptible haplotype, have been found to be predominantly associated with ACPA-positive RA, even if the association with ACPA-negative RA cannot be excluded.

We evaluated the influence of genes conferring susceptibility to RA on the production of anti-VCP antibodies and tested the interaction of VCP with HLA *in vitro*. We analyzed 172 patients with RA of French white origin[3]. All provided informed consent and the Ethics Committee of Hôpital Bicêtre (AP-HP, Paris, France) approved the study.

HLA-DRB1 typing and subtyping were performed by a polymerase chain reaction (PCR)-based method, using a panel of sequence-specific oligonucleotide probes. PADI4 haplotypes were obtained by typing 3 single-nucleotide polymorphisms (SNP: rs1886303, PADI4_92, PADI4_96 – CGC haplotype) by PCR-restriction fragment-length polymorphism (RFLP)[4]. PTPN22 (R620W; rs24766601 – T variant) and C5/TRAF1 region polymorphisms (rs10818488 – A variant) were genotyped by PCR-RFLP[5,6].

VCP1 (citrullinated $EBNA1_{35\text{-}58}$: GGDNHGCitGCitGCitGCit GCit-GGGCitPGAPG) and VCP2 (citrullinated $EBNA2_{338\text{-}358}$: GQSCitGQSCit-GCitGCitGCitGCitGCitGKG) were synthesized either as linear peptides (for the peptide-binding assay) or as multiple antigen peptides (for ELISA). Anti-VCP antibodies were detected by ELISA on VCP1 or VCP2 coated plates[2], setting the upper limit of normality at the 98.5 percentile of 100 normal controls. Anti-cyclic citrullinated peptide antibodies were detected using an anti-CCP2 kit.

Anti-VCP1 antibodies were detected in 78/172 (45%) patients with RA, anti-VCP2 in 106/172 (62%), and anti-CCP2 in 130/172 (76%). The presence of at least 1 SE allele was not associated with the presence of either anti-CCP2 or anti-VCP antibodies. By contrast, 2 copies of the HLA-SE alleles conferred an increased risk to produce anti-CCP2 ($p = 0.01$) or anti-VCP2 ($p = 0.05$) antibodies with respect to 1 or no SE allele.

Analyzing the contribution of individual SE alleles, the *0401 allele conferred an increased risk to produce anti-VCP2 ($p = 0.007$) and anti-CCP2 ($p = 0.02$) antibodies; *0404 was associated with the production of anti-VCP1 ($p = 0.05$) and anti-VCP2 ($p = 0.04$) antibodies (Table 1).

Patients were subdivided into 3 groups: those carrying no SE alleles (n = 34), 1 SE allele (n = 112), or 2 SE alleles (n = 26). The mean anti-VCP level increased as a function of the SE status; the level of anti-VCP2 antibodies was significantly higher in the double-SE patients compared to the negative patients ($p = 0.01$; Figure 1B). Anti-VCP1 antibodies were similarly higher in double-HLA-SE patients than in SE-negative patients, but for this there was only a trend to significance ($p = 0.06$; Figure 1A). Patients carrying *0401 or *0404 alleles had higher levels of anti-VCP1 ($p < 0.05$; Figure 1C, 1E) and anti-VCP2 antibodies ($p < 0.05$; Figure 1D, 1F). In contrast, PTPN22, TRAF1/C5, and PADI4 polymorphism did not influence the production or the level of anti-VCP or anti-CCP2 antibodies (Table 1).

Finally, competitive peptide binding assays were conducted to determine the affinity of VCP1 and VCP2 for purified MHC molecules (DRB1*0101, *0401, *0404, *0301, *0701, *0802, *1101, and *1302) as described[7,8]. The nanomolar concentration of unlabeled viral peptides necessary for 50% inhibition of the labeled peptide to the purified

Table 1. The association between genetic factors and ACPA was tested in 172 RA patients by comparing (Fisher exact test) the distribution of patients positive and negative for anti-VCP antibodies among carriers and noncarriers for each genetic variant.

Factor		Anti-VCP1				Anti-VCP2				Anti-CCP			
		Neg	Pos	OR (95% CI)	Fisher p	Neg	Pos	OR (95% CI)	Fisher p	Neg	Pos	OR (95% CI)	Fisher p
OR for developing anti-VCP antibodies in the presence of susceptible genes (HLA-SE, PTPN22, TRAF1.C5, PAD4)													
HLA-SE	No	21	12	1.646	0.23	15	18	1.409	0.42	11	22	1.862	0.17
	Yes	73	66	(0.734–3.687)		51	88	(0.642–3.092)		29	108	(0.810–4.278)	
PTPN22	No	66	48	1.473	0.25	44	70	1.028	1	30	84	1.036	0.45
	Yes	28	30	(0.780–2.780)		22	36	(0.536–1.974)		12	46	(0.64–2.092)	
TRAF1.C5	No	33	26	1.082	0.87	22	37	0.934	0.87	17	42	1.424	0.35
	Yes	61	52	(0.574–2.038)		44	69	(0.487–1.784)		25	88	(0.695–2.919)	
PAD4	No	26	22	1.807	0.2	28	20	1.108	0.83	38	10	1.005	1
	Yes	17	26	(0.784–4.163)		24	19	(0.482–2.545)		34	9	(0.365–2.768)	
OR for developing anti-VCP antibodies in the presence of HLA-DRB1 shared epitope alleles													
HLA-DRB*0101	No	66	63	0.561	0.15	45	84	0.561	0.1	31	98	0.92	0.83
	Yes	28	15	(0.273–2.975)		21	22	(0.279–1.129)		11	32	(0.415–2.038)	
HLA-DRB*0401	No	57	38	1.621	0.126	54	50	**2.4**	**0.007**	30	65	**2.5**	**0.02**
	Yes	37	40	(0.883–2.975)		21	56	**(1.261–4.566)**		12	65	**(1.177–5.306)**	
HLA-DRB*0404	No	88	65	**2.93**	**0.05**	63	90	**3.733**	**0.04**	40	113	3.008	0.16
	Yes	6	13	**(1.058–8.127)**		3	16	**(1.043–13.35)**		2	17	(0.665–13.60)	
HLA-DRB*0405	No	88	72	1.222	0.77	62	98	1.265	0.76	41	119	3.789	0.29
	Yes	6	6	(0.377–3.952)		4	8	(0.365–4.379)		1	11	(0.474–30.266)	
HLA-DRB*0408	No	90	74	1.216	1	63	101	1.039	1	40	124	0.967	1
	Yes	4	4	(0.294–5.029)		3	5	(0.240–4.501)		2	6	(0.187–4.986)	
HLA-DRB*0102	No	72	66	0.595	0.24	52	86	0.863	0.7	30	108	0.509	0.12
	Yes	22	12	(0.273–1.396)		14	20	(0.402–1.856)		12	22	(0.226–1.146)	
HLA-DRB*1002	No	66	63	0.561	0.15	45	84	0.561	0.1	31	98	0.92	0.83
	Yes	28	15	(0.274–1.148)		21	22	(0.279–1.129)		11	32	(0.415–2.038)	

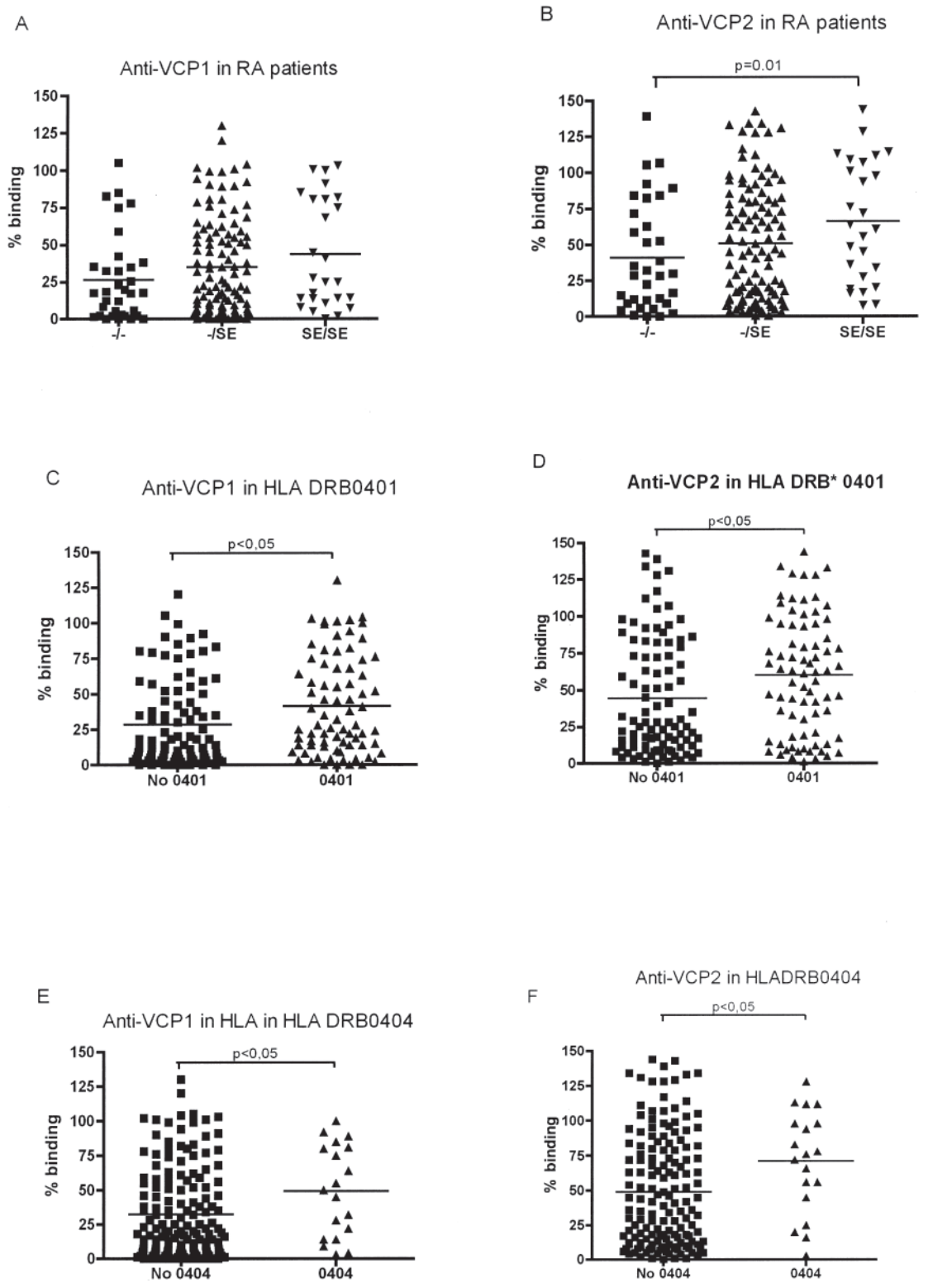

Figure 1. Levels of anti-VCP1 and anti-VCP2 antibodies were analyzed in patients with rheumatoid arthritis subdivided according to HLA-SE (A, B), HLA-DRB1*0401 (C, D), and HLA-DRB1*0404 status (E, F); Kruskal-Wallis test, with Dunn's multiple comparison test when appropriate and Mann-Whitney U test.

HLA-DRB1 molecules (IC_{50}) was used as an approximation of the affinity of interaction (*K*D), with expression of results as the inverse of the IC_{50} values measured in nanomolar concentration. Biologically relevant binding ($IC_{50} \leq 5000$ nM) was not detected with either peptide for any of the HLA class II molecules tested.

These results indicate that the immune response to citrullinated viral antigens is associated with SE alleles, and in particular with *0401 and *0404. A strong gene-dose effect was observed on both the production and the level of anti-VCP antibodies. Using competitive assays, no binding of the viral sequences to any SE allele was detected.

DRB1*0404 is associated with the production of anti-VCP antibodies and *0401 with anti-VCP2 and anti-CCP2. Similarly to our findings, it has been reported that *0401 and/or *0404 are associated with the production of anti-CCP, anti-citrullinated fibrinogen, and anti-citrullinated enolase peptide 1 antibodies[9].

We could not detect any influence of PTPN22, TRAF1/C5, and PADI4 on the immune response to VCP and CCP2. Lack of statistical power because of the small sample size is likely the cause of these negative findings; associations between these genes and anti-CCP have in fact been found in larger cohorts[10,11].

The molecular mechanisms underlying the effect of DRB1 on ACPA production are unknown. It has been proposed that the conversion of arginine into citrulline allows a high affinity interaction with the positively charged pocket of SE alleles. Experimental evidence in support of this hypothesis was obtained in the case of the *0401 allele, which interacted strongly *in vitro* with a citrullinated vimentin peptide, while the arginine-containing sequence has a low binding affinity[12]. It has been reported that purified DRB1 molecules bind to a similar extent to fibrinogen-derived peptides containing arginine or citrulline[13]. Those results, however, were obtained by a qualitative direct binding assay method with sensitivity not comparable to the competitive binding assay. In our study, no binding of citrullinated peptides to HLA alleles was detected by the same stringent competitive assay, with sensitivities in the nanomolar range used for the citrullinated vimentin peptide[12]. It is of interest that despite the association of anti-VCP2 and HLA-DRB*0401, no binding of the peptide to the purified HLA molecule was detected.

The immune response to VCP is under the genetic control of the *0401 and *0404 alleles, but these molecules do not bind the citrullinated peptides. Thus, the differential binding properties of DRB1* alleles for either arginine- or citrulline-containing sequences do not always explain the HLA control of the immune response to citrullinated epitopes.

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