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Detection of disease-specific restriction fragment length polymorphisms in pemphigus vulgaris linked to the *DQw1* and *DQw3* alleles of the *HLA-D* region

(HLA-disease associations/autoimmunity)

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ABSTRACT **Pemphigus vulgaris in Israeli Ashkenazi and non-Ashkenazi Jews and in Austrian non-Jewish patients is strongly associated with the *DR4* and *DRw6* alleles of the *HLA-D* region class II genes. Restriction fragment length polymorphism analysis was undertaken with DQβ, DQα, and DRβ cDNA probes. Hybridization with the DQβ probe identifies *Pvu* II, *Bam*HI, and *Eco*RV fragments that absolutely discriminate pemphigus vulgaris patients from healthy DR-, DQ-, and ethnic-matched controls. In contrast the DQα and DRβ probes failed to identify disease-specific restriction fragment length polymorphism fragments. These studies indicate that DQw1 and DQw3 polymorphisms carried by pemphigus vulgaris patients may be directly involved in predisposition to the disease or may be tightly linked to the susceptibility gene itself. To our knowledge, this is the first example of an HLA restriction fragment length polymorphism that is highly associated with susceptibility to autoimmune disease.**

Pemphigus vulgaris (PV) is a dermatological autoimmune disease mediated by autoantibodies directed against an antigen, intracellular cement substance, in the basement membrane of keratinocytes, resulting in blister formation (1, 2). This disease can be reproduced in monkeys or in mice after transfer of antibodies from PV patients (1, 2). Susceptibility to PV is strongly associated with serologically defined gene products of the *HLA-D* region (3, 4). The genes of the *HLA-D* region (*DR, DQ, DP*) code for the class II antigens. These antigens are transmembrane proteins that are expressed on certain cells involved in the immune response. The HLA antigens are highly polymorphic and are critical in several immune functions (5, 6). The strong association of the class II genes with PV, may thus indicate that these genes play a role in pathogenesis of this disease. We investigated the associations between PV and HLA class II antigens in Israeli Jewish and Austrian non-Jewish patients at both the serological and molecular levels. The results indicated that susceptibility to PV is in linkage disequilibrium with two alleles of the *DQβ* locus, namely *DQw1* and *DQw3*. Molecular analysis with a DQβ cDNA probe revealed disease-specific restriction fragment length polymorphisms (RFLPs) that discriminate patients from healthy controls. To our knowledge, this is the first example of RFLPs in the class II region of the *HLA* supergene family that identify susceptibility to an autoimmune disease.

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MATERIALS AND METHODS

Patients and Control Individuals. Thirty-seven Israeli Jewish patients (20 Ashkenazi and 17 non-Ashkenazi) were studied. The control group included 41 Ashkenazi and 35 non-Ashkenazi Jews, randomly selected healthy individuals. Forty-five DR- and DQ-matched individuals were selected as controls for RFLP analysis. In addition 16 non-Jewish Austrian PV patients were investigated and compared to randomly selected healthy Austrians.

Serologic Method. Human peripheral blood lymphocytes were isolated by flotation on Ficoll-Paque. HLA-A, -B, -C typing was performed by the National Institutes of Health standard complement-dependent microlymphocytotoxicity technique. HLA-DR and -DQ typing was done on T-cell-depleted B-cell-enriched lymphocytes by extended incubation cytotoxicity testing (7). The HLA antisera included alloantisera from the third Asia–Oceania Histocompatibility Workshop and sera of local origin.

DNA Extraction, Digestion, and Binding to Nylon Membranes. Genomic DNA was extracted from peripheral leukocytes. After lysis of the erythrocytes, the leukocytes were treated with proteinase K at 0.2 mg/ml (Boehringer Mannheim) for 12 hr at 42°C. Two phenol/chloroform-isoamyl alcohol, 3:1 (vol/vol), and two isoamyl alcohol/chloroform, 1:24 (vol/vol), extractions followed. Thereafter, the DNA was precipitated with two volumes of absolute ethanol. After precipitation and several washes with ethanol, the DNA was dissolved in TE (1 mM Tris·HCl/0.1 mM EDTA, pH 7.5) to give a final concentration of 1 mg/ml. Ten micrograms of each DNA sample was digested with 10 units of the following restriction enzymes: *Bam*HI, *Dra* I, *Eco*RI, *Eco*RV, *Hin*cII, *Hin*dIII, *Pst* I, *Pvu* II, and *Taq* I. Digestion was carried out for 15 hr, and the DNA samples were subjected to electrophoresis at 30 V in agarose gel (0.7% agarose in Tris acetate buffer, pH 6.4, containing ethidium bromide at 20 μg/ml) for 36 hr. Gels were then transferred to Hybond N membranes (Amersham) as described by Southern (8).

cDNA Probes and Hybridization. The DQα cDNA probe was furnished by Charles Auffray (College de France). The DQβ and DRβ cDNA probes were furnished by Hugh McDevitt and John Bell (Stanford University). Full descriptions of the probes appear elsewhere (9, 10). The probes were labeled by the nick-translation method (11) using [α-^{32}P]-dCTPand [α-^{32}P]dATP (3000 Ci/mmol, 1 Ci = 37 GBq, Amersham). Hybridization was carried out as follows: For prehybridization membranes were incubated for 24 hr at 42°C

Abbreviations: PV, pemphigus vulgaris; RFLP, restriction fragment length polymorphism.

in 50 mM Pipes, pH 7.5; 5× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7), 50% (vol/vol) formamide (deionized), 0.5% $NaDodSO_4$, 5× Denhardt's solution (12), and denatured salmon DNA at 100 μg/ml. Hybridization was carried out in the same solution but including 10% (wt/vol) dextran sulfate and the nick-translated probe (2×10^6 cpm/cm^2) in the same conditions but for 48 hr. After hybridization, the membranes were washed twice for 5 min in 2× SSC at room temperature, then once for 5 min in 0.1× SSC/0.1% $NaDodSO_4$ at room temperature. Thereafter, the membranes were dried at room temperature and exposed to Agfa Curix RP2 x-ray films.

RESULTS

Typing for the serologically defined class II HLA-DR and -DQ antigens revealed that two antigens, DR4 and DRw6, were present in nearly all patients with PV. Thirty-five of 37 patients had DR4, DRw6, or DR4 and DRw6 antigens (DR4,DRw6) (Table 1). For Israeli Ashkenazi PV patients 19 of 20 patients had DR4 antigen (of whom 5 had DR4,DRw6) compared to 16 of 36 control individuals who carried DR4 (of whom 3 had DR4,DRw6) [RR (relative risk) = 24; $\chi^2 = 12$]. The remaining patient had DRw6. For Israeli Jewish non-Ashkenazi patients, DR4 was present in 5 of 17 patients, DR4 and DRw6 were in 4 of 17 patients, and DRw6 was in 6 of 17 patients. The remaining two patients had DR5,DR$^-$ and DR5,DR7. In non-Jewish Austrian PV patients 15 of 16 had either DR4 (5 of 16), DRw6 (5 of 16), or DR4 and DRw6 (5 of 16). One Austrian patient was DR5,DR7 (Table 1).

In general, DR4 individuals always had DQw3 antigen, whereas DRw6 individuals had either DQw1 or DQw3. As expected all DR4 PV patients had DQw3. All 26 PV patients who had DRw6 also had DQw1. One DRw6 patient had both DQw1 and DQw3.

PV thus represents a disease with nearly 100% association with either of two HLA-D alleles, *DR4* or *DRw6*. Only two other diseases, narcolepsy, associated with the *HLA-DR2* allele (13), and celiac disease associated with the *DQw2* allele (14), show such a strong association with the *HLA-D* region. In view of these results, we asked whether the *DR4* and *DRw6* alleles in PV patients were distinct from alleles in healthy individuals, and, by applying molecular biological approaches, whether it would be possible to obtain RFLPs that could discriminate PV patients from healthy individuals.

RFLP Analysis with the DQβ cDNA Probe of Israeli PV Patients and Matched Healthy Individuals. DNA from 37 PV patients and 45 DR$^-$ and DQ$^-$ matched healthy control individuals (also matched for ethnicity) was digested with several restriction enzymes. *Eco*RI, *Eco*RV, *Pst* I, *Hin*cII, *Bam*HI and *Pvu* II gave highly informative fragments (Table 2). Table 2 is stratified so that each RFLP is analyzed with controls matched for ethnicity and HLA-DR and -DQ type. In addition homozygous typing cells of unknown ethnic origin, but HLA-DR and -DQ matched are also shown. Except for the 8.3-kilobase (kb) *Bam*HI polymorphism, there were no differences in the frequency of informative bands when comparing Ashkenazi and non-Ashkenazi populations.

Table 1. Joint occurrence of DR4 and DRw6 in PV patients

		Individuals carrying the haplotype, no.					
		Ashkenazi		Non-Ashkenazi		Austrian	
DR4	DRw6	PV	Control	PV	Control	PV	Control
+	+	5 (25)	3 (7)	4 (23)	2 (6)	5 (31)	1 (6)
+	−	14 (70)	13 (32)	5 (29)	7 (20)	5 (31)	2 (12)
−	+	1 (5)	12 (29)	6 (35)	11 (31)	5 (31)	3 (19)
−	−	0	13 (32)	2 (12)	15 (43)	1 (6)	10 (62)

Values in parentheses are percent of total number. +, Presence of marker; −, absence of marker.

In DRw6 individuals with PV, the DQβ probe hybridized with a 2.5-kb *Bam*HI fragment in 16 of 16 patients and in only 1 of 16 matched healthy individuals (Fig. 1). A 5.3-kb *Pst* I fragment also discriminated DRw4,DRw6 PV patients (9 of 9) from control individuals (0 of 4). In DR4 PV patients, a *Pvu* II 6.9-kb fragment hybridized with the DQβ probe in 5 of 6 DR4,DR$^-$ patients; in 6 of 6 DR4,DR5 patients; in 0 of 13 DR4,DR$^-$ healthy controls; and 1 of 6 DR4,DR5 healthy control individuals. An additional 12-kb *Pst* I fragment discriminated DR4,DR5 patients (6 of 6) from control individuals (1 of 6). An 8.3-kb RFLP appeared after *Bam*HI digestion in 6 of 6 DR4,DR1 PV patients and in 2 of 5 DR4,DR1 control individuals. The 2.5-kb *Bam*HI fragment that discriminated all DRw6 PV patients from control individuals not only appeared in all six DR4,DR1 PV patients but also appeared in some (2 of 5) of the matched DR4,DR1 control individuals. It is noteworthy that the 6.9-kb *Pvu* II fragment present in 11 of 12 DR4,DR$^-$ and DR4,DR5 Israeli PV patients, was absent from all 6 Israeli PV patients carrying the DR4,DR1 haplotype. RFLPs seen in normal individuals and never in HLA-DR, -DQ matched PV patients were also observed. A 6.1-kb *Eco*RV fragment hybridizing with the DQβ probe was seen in 15 of 16 DRw6 healthy individuals, but was absent in all 16 DRw6 PV patients. A 2.2-kb *Hin*cII fragment hybridizing with the same probe was seen in 5 of 6 DR4,DR5 control individuals and 0 of 6 DR4,DR5 PV patients.

In Table 2 various restriction fragments hybridizing with the DQβ probe are shown that either absolutely or with rare exception discriminate between individuals with disease and those who are healthy. It is noteworthy that for different HLA-DR haplotypes, specific restriction fragments are informative. Thus, a *Pvu* II 6.9-kb fragment discriminates HLA-DR4,DR$^-$ and HLA-DR4,DR5 PV patients from control individuals, whereas a *Bam*HI 2.5-kb fragment discriminates DR4,DRw6; DR5,DRw6; DRw6,DR$^-$; and DRw6,DR7 PV patients from control individuals. Similarly a variety of RFLPs distinguish healthy individuals absolutely from PV patients. For example, an *Eco*RV 6.1-kb fragment is present in healthy individuals who are HLA-DR4,DRw6; DR5, DRw6; DRw6,DR$^-$; and DRw6,DR7 but not in PV patients carrying matched alleles. These discriminatory RFLPs are disease specific and appear to correlate with the serologic DQ type, not the DR type. The 6.9-kb *Pvu* II fragment appears in 11 of 12 DR4,DR$^-$ and DR4,DR5 patients; and in most (7 of 9) of the DR4,DRw6 patients. This fragment probably identifies a *DQw3* split. Twenty-two of 24 patients carrying the *Pvu* II 6.9-kb band had the DQw3 antigen. The *Bam*HI 2.5-kb fragment is present in all 22 PV patients carrying either the *DR1* or *DRw6* alleles. This fragment identifies a *DQw1* split.

One of the PV patients had DR5,DR$^-$ and one had DR5,DRw7. The DR5,DR$^-$ patient possessed the 6.9-kb *Pvu* II fragment whereas the other patient had no informative RFLP.

An additional *Pvu* II 4.1-kb fragment appeared in almost all patients (36 of 37) and in about half of the control individuals (26 of 45) (Table 2). Additional fragments appearing in most of the healthy DRw6 and DRw5 individuals and never seen in PV patients were also encountered with the following enzymes *Bam*HI (Fig. 1), *Eco*RI, *Dra* I, *Pst* I, and *Pvu* II (results not shown).

RFLP Analysis with DQβ cDNA Probe in Austrian PV Patients. Sixteen Austrian PV patients were tested. Serologic typing revealed the following antigens: DR4,DRw6 (5 patients), DR4,DR$^-$ (2 patients); DR4,DR5$^-$ (2 patients); DR5, DR6$^-$ (2 patients); DR4,DR1$^-$ (1 patient); DR6,1$^-$ (1 patient); DR6,$^-$ (1 patient); DRw6,DR7$^-$ (1 patient); and DR5,DR7,$^-$ (1 patient). In this population, PV patients carrying the DRw6

Table 2. Disease-specific RFLPs in Israeli Jewish PV patients

Restriction fragment	Phenotype DR	Phenotype DQ	No. containing RFLP/total no. tested: Ashkenazi individuals PV	Ashkenazi individuals C	Non-Ashkenazi individuals PV	Non-Ashkenazi individuals C	HTC C	Total PV Patients	Total C	χ^2 (df = 1)
*Bam*HI (2.5 kb)	DRw6,DRX	DQw1	6/6	0/6	10/10	1/8	0/2	16/16	1/16	24.60
*Eco*RI (14 kb)	DRw6,DRX	DQw1	6/6	0/6	8/10	1/8	0/2	14/16	1/16	18.07
Pst I (5.3 kb)	DR4,DRw6	DQw3,DQw1	5/5	0/1	4/4	0/3	–	9/9	0/4	8.73
*Eco*RV (6.1 kb)	DRw6,DRX	DQw1	0/6	6/6	0/10	7/8	2/2	0/16	15/16	24.60
Pvu II (6.9 kb)	DR4,DR$^-$	DQw3	3/3	0/6	2/3	0/3	0/4	5/6	0/13	19.64
	DR4,DR5	DQw3	4/4	1/3	2/2	0/3	–	6/6	1/6	
Pst I (12 kb)	DR4,DR5	DQw3	4/4	1/3	2/2	0/3	–	6/6	1/6	5.49
*Hinc*II (2.2 kb)	DR4,DR5	DQw3	0/4	2/3	0/2	3/3	–	0/6	5/6	5.49
*Bam*HI (8.3 kb)	DR1,DR4	DQw1,3	6/6	1/3	0/0	1/2	–	6/6	2/5	2.86
Pvu II (4.1 kb)	All	DQw1,3	20/20	12/21	16/17	9/18	4/4 DR4 1/2 DR6	36/37	25/45	16.44

HTC, homozygous typing cells; C, control individuals or cells; –, not done.

haplotype were distinguished by the 2.5-kb *Bam*HI fragment that was present in 8 of 10 patients and in 0 of 3 DRw6 Austrian control individuals. The 6.9-kb *Pvu* II fragment was present in all (10 of 10) Austrian PV patients who were DR4 carriers (Fig. 2), including a DR4,DR1 carrier. This fragment was not seen in the 6 Israeli PV patients with the DR4,DR1 haplotype. This Austrian patient also showed the *Bam*HI 2.5-kb fragment (seen in Israeli DR4,1 patients) associated with *DQw1* allele. Another patient carried a DR5,DR7 haplotype and, similar to the Israeli Jewish DR5,DRw7 PV patient, this individual did not have any PV-associated RFLP fragments.

RFLP with DRβ and DQα cDNA Probes in the Israeli PV Patients and Matched Controls. The same membranes used for the detection of RFLP and the DQβ probe were hybridized with the DRβ and DQα probes. No disease-specific RFLP fragments for PV were detected with either DRβ or DQα cDNA probes.

DISCUSSION

The serologic data demonstrated that PV in Israeli Jewish and Austrian non-Jewish populations is predominantly associated with the DR4 and DRw6 haplotypes. The RFLP analysis clearly revealed that the association is not with the *DRβ* region but rather with the *DQβ* region, and in particular with either the *DQw1* and *DQw3* alleles. A *Pvu* II 6.9-kb fragment discriminated DR4, DR$^-$, and DR4,DR5 PV patients from healthy individuals, while a *Bam*HI 2.5-kb fragment discriminated DRw6 PV patients from healthy individuals. Both of these highly informative RFLPs were seen in comparable frequency in the Ashkenazi and non-Ashkenazi populations. In addition 8.3-kb *Bam*HI fragment discriminates DR4,DR1 PV patients from control individuals. The 2.5-kb *Bam*HI fragment most probably represents a *DQw1* polymorphism whereas the 6.9-kb *Pvu* II fragment represents a *DQw3* polymorphism. The fact that three patients who did not carry DR4 or DRw6 were positive for the *DR5* allele could be explained on the basis that they carried DQw3.

The *Bam*HI, *Eco*RV, *Eco*RI, *Hinc*II, *Dra* I, and *Pvu* II restriction fragments appearing in almost all of the healthy individuals, but not in PV patients, also correlated with the DQw1 and DQw3 haplotypes. This finding supports the hypothesis that PV is associated with the *DQw1* and *DQw3* alleles. Because RFLP fragments that hybridize to the DQβ probe discriminated PV patients from matched control indi-

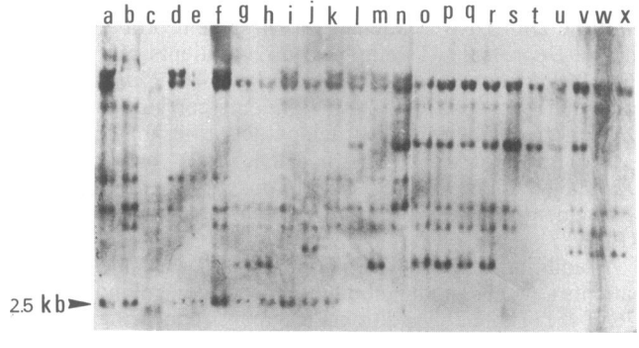

FIG. 1. *Bam*HI 2.5-kb band hybridizing with DQβ probe in Israeli PV patients and control individuals. Lanes a–j are PV patients; lanes k–x are healthy controls. Lanes: a–f, DR4,DR6; g, DR5,DR6; h, DR6,DR$^-$; i and j, DR6,DR7; k–n, DR4,DR6; o–r, DR5,DR6; s–u, DR6,DR$^-$; v and w, DR6,DR7; x, a DR7 homozygous typing cell. A *Bam*HI 5.8-kb band is present in most control individuals, but never in PV patients. This band was found in two of four healthy DR4,DR6 individuals but in none of the 9 DR4,DR6 PV patients. The 5.8-kb band was seen in all five healthy DR5,DR6 individuals and never in four DR-matched PV patients. The band was absent in one DR6,DR$^-$ PV patient and present in three of four matched healthy individuals.

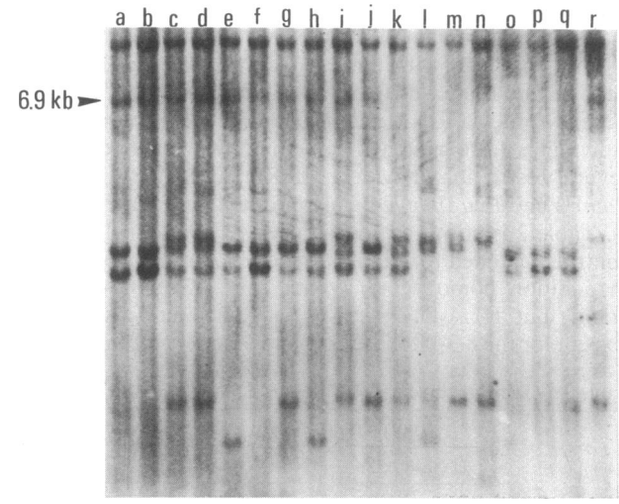

FIG. 2. A *Pvu* II 6.9-kb band hybridizing with DQβ probe in Austrian PV patients (lanes a–i, k, l, and n–q) and selected Austrian control individuals (lanes j, m, and r). Lanes: a, DR4,DR$^-$ patient; b, DR4,DR1 patient; c and d, DR4,DR5 patient; e–i, DR4,DR6 patient; j, DR4,DR6 control individual; k and l, DR5,DR6 patient; m, DR5,DR6 control; n, DR5,DR7 patient; o, DR6,DR$^-$ patient; p, DR6,DR1 patient; q, DR6,DR7 patient; r, DR3,DR4 control.

viduals and because no disease-specific RFLP was found for PV with the DRβ and DQα probes, the *DQβ* gene and specifically polymorphisms in the *DQw1* and *DQw3* alleles may be associated with susceptibility to PV. Although the dramatic RFLP associations reported here may well reflect *DQβ* susceptibility sequences, given linkage disequilibrium, they are also consistent with susceptibility sequences in linked loci (e.g., *DRβ* or *DX*). Sequence studies will reveal the actual polymorphic regions, which might be within *DQ* or in neighboring class II genes. It is noteworthy that DR2, DRw8, and DRw10 haplotypes, which are also in linkage disequilibrium with the *DQw1* allele, were not found in any of the PV patients. In addition it is interesting to note that the *DR3* allele associated with *DQw2*, which is linked with several autoimmune disorders (14), was absent from all PV patients studied.

RFLP analysis has been used to demonstrate associations between *HLA-DR* and insulin-dependent diabetes mellitus and multiple sclerosis (15) and between *HLA-DQ* and myasthenia gravis (16), insulin-dependent diabetes (17), juvenile rheumatoid arthritis (18), celiac disease (19), and rheumatoid arthritis (20). These studies have revealed strong associations between certain RFLPs and disease. However, in most of these studies the association between RFLP and disease has been far from absolute. For instance, in the study linking an *HLA-DQ* gene RFLP and myasthenia gravis, fewer than half of the DR3 myasthenia gravis patients carried the disease-related polymorphism (16). On the other hand a very strong association was noted in celiac disease where a 4.0-kb *Rsa* I fragment was detected with a DQβ probe in 18 of 20 DQw2 patients and 2 of 11 DQw2 control individuals (19). Even stronger associations were seen in the PV patients, with RFLP markers that give absolute discrimination between healthy individuals and those with the disease.

Why has the study of PV given such striking results, while other studies on insulin-dependent diabetes, multiple sclerosis, and myasthenia gravis have revealed less-informative associations. Perhaps PV is most amenable for the RFLP approach because the serologic studies show a nearly 100% association with two HLA-D determinants. In only two other diseases, narcolepsy with the *HLA-DR2* gene and celiac disease with the *DQw2* allele, are the associations as strong. It is noteworthy that of the 2 exceptions out of 37 PV patients who did not have HLA-DR4 or -DRw6, one patient, a DR5,DR$^-$, had RFLPs shared with PV patients who had DR4. The other patient who had HLA-DR5,DR7 had distinctive clinical symptoms with systemic lupus erythematosus, myasthenia gravis, and insulin-dependent diabetes in addition to PV. This individual had RFLPs not seen in other PV patients or control individuals.

The discovery of disease-specific RFLPs associated with HLA class II molecules in PV will allow us to investigate further how these genes promote susceptibility to autoimmune disease. Because the critical autoantibody and autoantigen is known for PV, a situation that exists for only a few other autoimmune conditions in man—for example, myasthenia gravis and celiac disease—we are now in the position to analyze the molecular and immunogenetic role of HLA in this autoimmune disease. These findings will allow the identification of the precise PV-susceptibility genes in the HLA-class II region.

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