An HLA Class II Region Restriction Fragment Length Polymorphism (RFLP) in Patients with Dermatitis Herpetiformis: Association with HLA-DP Phenotype

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Dermatitis herpetiformis (DH) is characterized in part by an associated gluten-sensitive enteropathy (GSE), and a strong association with the HLA antigens HLA-A1, -B8, -DR3, and -DQw2, essentially identical to that seen in patients with isolated GSE (celiac disease). A 4.0-kb RsaI RFLP has been identified using a DQ β-chain cDNA and localized to the HLA-DP β-chain region. This RFLP has been found more frequently in patients with isolated GSE than in normal HLA matched controls. We have analyzed genomic DNA from 24 patients with DH and 15 HLA-matched controls to determine if this 4.0-kb RsaI RFLP was present in patients with DH. Twenty-one of 24 (87%) of patients with DH were found to have this RFLP as compared to 7 of 10 (70%) HLA-DR3, -DQw2 matched control subjects ($p = 0.23$). Thus, the 4.0-kb RsaI RFLP detected in patients with isolated GSE is also present in patients with DH; however, its frequency in DH patients does not differ significantly from that of HLA matched controls. Family studies of patients with DH revealed that although the 4.0-kb RsaI RFLP segregated with the HLA-A1, -B8, -DR3, -DQw2 haplotype in one family, it did not segregate with this disease-associated haplotype in two other families. In both patient and control populations, this RFLP was associated with HLA-DPw1 or -DPw3 phenotypes; 25 of 26 (96%) HLA-DPw1 or -DPw3 subjects were found to have this RFLP compared to only 1 of 6 (17%) who did not express HLA-DPw1 or -DPw3 ($p_c = 0.0009$). These population and family data suggest that this 4.0-kb RsaI RFLP is primarily associated with the HLA-DPw1, -DPw3 phenotype, rather than the clinical manifestations of DH. These data further document that the strongest association of DH with HLA antigens remains with HLA-DQw2 and HLA-DR3 antigens. *J Invest Dermatol 95:172–177, 1990*

Dermatitis herpetiformis (DH) is a chronic, blistering skin disease that is characterized in part by the presence of IgA deposits at the dermal–epidermal junction, and an associated, often asymptomatic, gluten-sensitive enteropathy (GSE) [1,2]. Patients with DH also have been found to have an increased frequency of the HLA antigens HLA-A1, -B8, -DR3, and -DQw2, similar to that seen in patients with isolated GSE (celiac disease) [3–14]. Although 80–90% of patients with DH express HLA-A1 and HLA-B8 (normal subjects: HLA-A1, 24%; HLA-B8, 21%), in both patients with DH and with isolated GSE an even stronger association has been demonstrated with the HLA class II antigens HLA-DR3 and HLA-DQw2 (DH: HLA-DR3, 95%; HLA-DQw2, 100%; normal subjects: HLA-DR3, 23%; HLA-DQw2, 40%) [4,6–8,11]. Further analysis of these HLA associations has demonstrated that the increased frequency of the HLA class I antigens, A1 and B8, in patients with DH is primarily due to the strong positive linkage disequilibrium between HLA-A1 and -B8 and the HLA class II antigens HLA-DR3 and HLA-DQw2. Finally, analysis of a third region of expressed HLA class II genes, HLA-DP, has revealed no statistically significant increase in HLA-DP antigen frequency in patients with DH compared to HLA-DR3, -DQw2 matched control subjects [7]. Taken together, these data suggest that the HLA-DR and HLA-DQ regions, specifically HLA-DR3 and -DQw2, are most important in the pathogenesis of DH [4,7,8].

Although the clinical features of patients with DH and those with isolated GSE are readily distinguishable, the HLA antigen frequency of occurrence of the HLA-A1, -B8, -DR3, and -DQw2 antigens is almost identical in these two groups of patients [4,5,7]. Patients with isolated GSE have been reported to have a higher frequency of a 4.0-kb restriction fragment length polymorphism (RFLP) at the HLA-DP β-chain gene locus than normal HLA-DR3 control subjects [15]. In order to determine whether this 4.0-kb RFLP might provide a means of genetically distinguishing between patients with isolated GSE and those with DH, we analyzed genomic DNA of patients with DH to determine if the 4.0-kb HLA-DPβ region RFLP is present more frequently in patients with DH than in HLA-DR3, -DQw2 control subjects. In addition, we have

Manuscript received November 28, 1989; accepted for publication April 3, 1990.

Supported by grants from the U.S. Public Health Service: 5RO1-AM34718 (RPH), AM38474 (RJW), GM10356 (FEW), and the Veterans Administration (RPH).

Presented in abstract form at the Society for Investigative Dermatology, May 1989.

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Abbreviations:
DH: dermatitis herpetiformis
GSE: gluten sensitive enteropathy
HLA: human histocompatibility leukocyte antigens
RFLP: restriction fragment length polymorphism

0022-202X/90/$03.50

studied families of patients with DH to determine if this 4.0-kb RsaI RFLP segregates with the clinical manifestations of DH and the HLA-A1, -B8, -DR3, -DQw2 haplotype associated with DH. Finally, we have correlated the presence of this RFLP with HLA phenotype to determine if this 4.0-kb RsaI RFLP may be related to a particular HLA phenotype.

MATERIALS AND METHODS

Patient and Control Cell Lines Twenty-four patients with dermatitis herpetiformis were studied. All patients had typical clinical and histologic features of DH; and direct immunofluorescence revealed granular deposits of IgA at the dermal–epidermal junction of perilesional skin. Ten unaffected family members of five patients with DH were also studied. The HLA type of 18 of the DH patients and of all family members was determined as previously described for HLA class I antigens A and B and the HLA class II antigens DR, DQ, and DP [7]. The remaining six patients with DH were HLA-types at the HLA-A, -B, -DR, and -DQ loci as previously described [16]. Lymphoblastoid cell lines were established for all DH patients and all family members by culturing 2×10^6 peripheral blood mononuclear cells with the supernatant from the Epstein–Barr virus (EBV) producing cell line B95-8 in the presence of Cyclosporine A (2 μg/ml). The established B cell lines were maintained in RPMI-1640 with 0.1 mM nonessential amino acids, 2 mM glutamine, 1 mM pyruvate, 2×10^{-3} u/ml insulin, and 20% heat-inactivated fetal calf serum.

EBV-transformed cell lines from 10 HLA-DR3 and -DQw2 controls were studied as were five HLA-DP-typed control cell lines (ASHI Repository, Boston, MA).

DNA Purification Genomic DNA was prepared by incubating 50×10^6 of the EBV-transformed B cells at 55°C in 2 ml of a buffer containing 50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 0.5% SDS, and 0.1 mg/ml proteinase-K, pH 7.5, for 16 h. The solution was then extracted two times with Tris-HCl saturated phenol, followed by two extractions with chloroform/isoamyl alcohol (24:1, v:v). The solution was dialyzed against 10 mM Tris-HCl, 1 mM EDTA, pH 7.4 (TE). After dialysis one ninth the total volume of 3 M sodium acetate was added, followed by absolute ethanol 2.5 times the aqueous volume at −20°C. After incubation for 1 h at −20°C, the precipitated DNA was washed in 70% ethanol, and resuspended in TE buffer.

RFLP Analysis Ten micrograms of genomic DNA from patients, family members, and controls was digested for 4 h at 37°C with 50 U of the restriction endonuclease RsaI (Bethesda Research Laboratories, Gaithersburg, MD), according to the manufacturer's specifications. After digestion, the reaction was terminated by heating at 70°C for 5 min. The digested DNA was then separated by electrophoresis in a 0.8% agarose gel in 40 mM Tris/acetate, 1 mM EDTA, pH 7.8 buffer [17]. After electrophoresis, the DNA was denatured by washing the gel 30 min in a 0.5 M NaOH, 1.5 M NaCl buffer followed by three washes of 20 min each in a 0.025 M NaH_2PO_4, 0.025 M Na_2HPO_4, 0.7 H_2O buffer. The DNA was then transferred to Gene-screen plus (New England Nuclear, Wilmington, DE), as described by Southern, using 10 × SSC (1 × SSC = 150 mM sodium chloride, 15 mM sodium citrate, pH 7.4) for 16 h [17,18]. After transfer the Gene-screen plus was allowed to air dry and then was prehybridized for 6 h at 42°C in a buffer containing 50% deionized formamide, 10% dextran sulfate, 0.2% bovine serum albumin, 0.2% polyvinylpyrrolidone, 0.2% ficoll, 50 mM Tris, 0.1% sodium pyrophosphate, 1% SDS, and 100 μg/ml denatured salmon sperm DNA (Sigma Chemical, St. Louis, MO). The cDNA probe used for hybridization was a full-length cDNA encoding a DQβ chain (pII β-1) (provided by Dr. Per Peterson, (Scripps Research Foundation, La Jolla, CA) [19]. The probe was radiolabeled by the method of Feinberg and Vogelstein [20] and hybridization conducted overnight at 42°C after addition of ^{32}P-labeled probe to the prehybridization buffer to a final concentration of $1-2 \times 10^6$ cpm/ml. After hybridization, the filters were washed two times at room temperature in 2 × SSC for 5 min each, followed by two washes in 2 × SSC with 1% SDS at 62°C for 30 min each, followed by two washes in 0.1 × SSC at room temperature for 30 min each. The filters were then exposed to Kodak X-OMAT AR x-ray film with intensifying screens for 2–4 d.

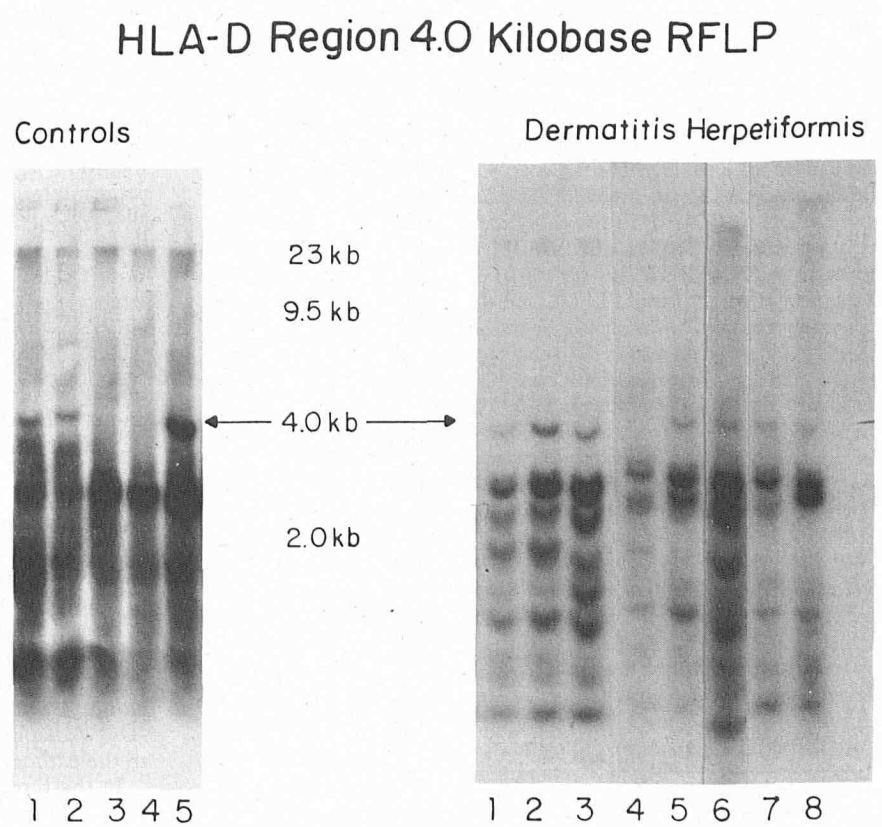

Figure 1. Southern blot analysis of RsaI digest of genomic DNA from eight patients with dermatitis herpetiformis and five control subjects, using the DQ β-chain cDNA probe pII β-1. The 4.0-kb RFLP *(arrows)* is seen in three of five control subjects and seven of eight patients with DH. Numbering of patients and controls corresponds to Table I.

RESULTS

RFLP Analysis DNA from 24 patients with DH was digested with the restriction enzyme RsaI and analyzed for the presence of a 4.0-kb RFLP using the DQ β-chain cDNA (pII β-1). Twenty-one of 24 (87%) patients with DH were found to have this 4.0-kb RsaI RFLP as compared to 7 of 10 (70%) HLA-DR3, -DQw2 control subjects (Fig 1) (Table I) ($p = 0.23$, Fisher's exact test). These data demonstrate that this 4.0-kb RsaI RFLP, although present in most patients with DH, is not specific for DH since it is also frequently present in the HLA matched (HLA-DR3, -DQw2) control population. Although this RFLP is not specific for DH, it may represent another marker of the HLA haplotype thought to be important in DH (HLA-A1, -B8, -DR3, -DQw2).

In order to determine if this RFLP segregates with the haplotype thought to play a role in the pathogenesis of DH (i.e., HLA-A1, -B8, -DR3, -DQw2, "DH haplotype"), five families containing 10 unaffected first-degree relatives and five patients with DH were analyzed for the presence of the 4.0-kb RsaI RFLP (Fig 2). The 4.0-kb RFLP was found in all five families studied; however, the data from two of these families (families 4 and 5, Table II) were not informative regarding the linkage of this 4.0-kb RsaI RFLP and the HLA-A1, -B8, -DR3, -DQw2 haplotype.

Table I. Patient and Control Population

	HLA-DPw	HLA-DQw	HLA-DR	Presence of 4.0-kb RsaI RFLP
Patients				
1	ND[a]	2[b]	3,7	+
2	ND	1,2	1,3	+
3	1,4	2,3	3,4	+
4	3,4	2,3	3,5	−
5	1,4	1,2	3,6	+
6	2,3	2,3	3,5	+
7	1,4	2,3	3,4	+
8	4	1,2	2,3	+
9	2,4	2,3	3,4	−
10	1,2	1,2	3,6	+
11	3,4	1,2	2,3	+
12	1,4	2	3,7	+
13	1,3	1,2	3,6	+
14	1,3	2	3	+
15	2,3	2	3,7	+
16	3	2	3,8	+
17	2	2	3,7	−
18	2,3	2	3,7	+
19	3,4	1,2	4,6	+
20	3	2	3,8	+
21	ND	2	3,7	+
22	ND	1,2	3,6	+
23	ND	2	3,7	+
24	ND	2,3	5,7	+
Controls				
1	ND	2	3,7	+
2	ND	2,3	3,12	+
3	ND	2,2	3,3	−
4	ND	1,2	2,3	−
5	ND	1,2	3,14	+
6	ND	2,2	3,7	−
7	ND	2,6	3,7	+
8	ND	2,3	3,4	+
9	ND	2	3,7	+
10	ND	1	3,8	+
11	3,3	5,5	16,16	+
12	3,3	7,7	11,11	+
13	3,3	6,6	13,13	+
14	1,1	5,5	1,1	+
15	1,1	6,6	13,13	+

[a]ND = Not done.
[b]HLA antigen.

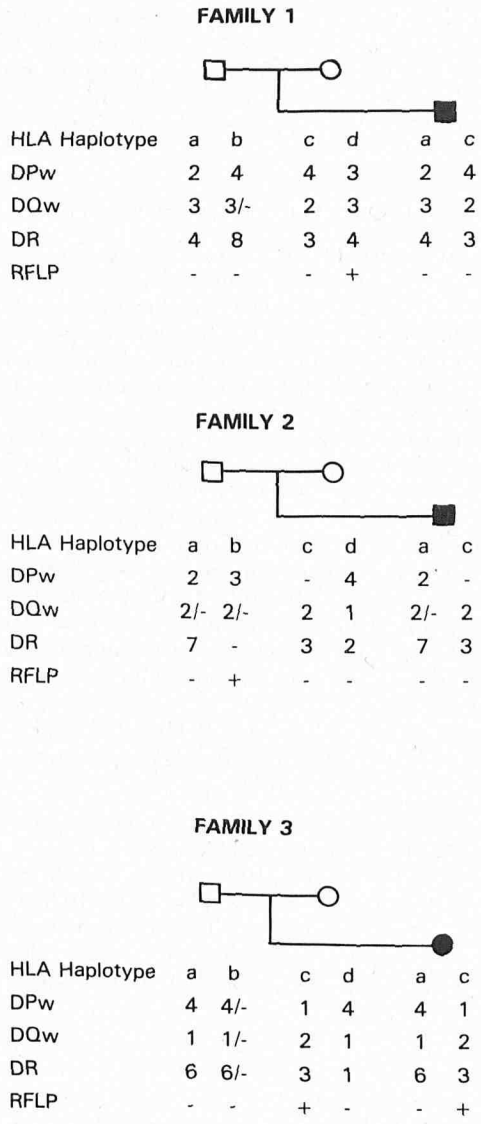

Figure 2. Family studies in patients with dermatitis herpetiformis showing the segregation of the 4.0-kb RsaI RFLP with HLA haplotypes. The 4.0-kb RsaI RFLP segregates with the HLA-DPw1 or -DPw3 phenotype and not necessarily with the haplotype most often associated with DH (HLA-A1, -B8, -DR3, -DQw2). Family numbers correspond to those shown in Table II.

In the remaining families, the RFLP data were informative. In one family (family 3, Table II), the RFLP did segregate with the HLA-A1, -B8, -DR3, -DQw2 haplotype. In the remaining families, however, this was not confirmed. In two families (families 1 and 2, Table II), the RFLP was not present in the patient with DH but rather was detected in an unaffected parent and did not segregate with the HLA-A1, -B8, -DR3, -DQw2 haplotype. This data demonstrates that the 4.0-kb RsaI is not in a strong positive-linkage disequilibrium with either the HLA-A1, -B8, -DR3, -DQw2 haplotype or the clinical manifestations of DH. This conclusion is further supported by the fact that an unaffected sibling of the patient in family 5 was also found to have the RFLP without any clinical evidence of DH and without having the HLA-A1, -B8, -DR3, -DQw2 haplotype. Thus, although this RFLP may often be present in patients with DH, it is not highly associated with either the clinical manifestations of the disease or the HLA-A1, -B8, -DR3, -DQw2 haplotype; and, therefore, is most likely not directly related to the pathogenesis of the disease.

In the three families where the RFLP could be assigned to an HLA haplotype (families 1, 2, and 3, Table II), the RFLP segregated

Table II. Family Studies of Patients with Dermatitis Herpetiformis

Family	Relation	Haplotype	DPw	DQw	DR	B	A	4.0-kb RFLP
1								
	Patient	a	2[a]	3	4	44	2	−
		c[b]	4	2	3	8	1	−
	Father	a	2	3	4	44	2	−
		b	4	3/−	8	44/−	3	−
	Mother	c[b]	4	2	3	8	1	−
		d	3	3	4	35	3	+
2								
	Patient	a	2	2/−	7	14	11	−
		c[b]	−	2	3	8	1	−
	Father	a	2	2/−	7	14	11	−
		b	3	2/−	7/−	27	2	+
	Mother	c[b]	−	2	3	8	1	−
		d	4	1	2	62	2	−
3								
	Patient	a	4	1	6	39	2	−
		c[b]	1	2	3	8	1	+
	Father	a	4	1	6	39	2	−
		b	4/−	1/−	6/−	27	28	−
	Mother	c[b]	1	2	3	8	1	+
		d	4	1	1	35	3	−
4								
	Patient	a	2	2/−	7	8	2	+/−
		c[b]	3	2	3	8	1	(+)
	Father	a	2	2/−	7	8	2	+/−
		b	4	2/−	3	50	11	+/−
	Mother	c[b]	3	2	3	8	1	(+)
		d	4	3	4	62	2	(+)
5								
	Patient	c[b]	1	2	3	8	1	(+)
		a	3	1	6	40	24	(+)
	Sister	b	4	2	7	62	2	(+)
		d	3	3	4	35	31	(+)
	Mother	c[b]	1	2	3	8	1	(+)
		d	3	3	4	35	31	(+)

[a] HLA antigen.
[b] Haplotype-presumed to encode disease gene(s) due to expression of HLA-A1, -B8, -DR3, -DQw2; − = undefined allele; x/− = uncertainty whether the haplotype expresses the allele "x" or an undefined allele; +/− = indeterminate presence of RFLP; (+) = RFLP present, but could not be assigned to specific haplotype.

with the HLA-DPw1 or -DPw3 phenotype and not necessarily with HLA-DR3 or -DQw2. To determine whether this RFLP might be associated with HLA-DPw1 and/or -DPw3, 3 homozygous HLA-DPw3 cell lines and 2 homozygous HLA-DPw1 cell lines (none of which expressed either HLA-DR3 or -DQw2) were analyzed for the presence of the 4.0-kb RsaI RFLP. In all five of these cell lines, the 4.0-kb RsaI RFLP was detected (Table I), supporting the hypothesis that this 4.0-kb RsaI RFLP may be associated with HLA-DPw1 and -DPw3. Analysis of all subjects (patients, unaffected family members, and controls) revealed that the 4.0-kb RsaI RFLP was present in 25 of 26 (96%) HLA-DPw1 or -DPw3 subjects, but in only 1 of 6 (17%) subjects who did not express HLA-DPw1 or -DPw3 ($p_c = 0.0009$, two-tailed Fisher's exact test corrected for 5 HLA-DP alleles tested for).

DISCUSSION

The distinctiveness of the clinical features of DH and isolated GSE suggests that each disease may have unique genetic characteristics that would separate patients with DH from those with isolated GSE. In general, however, it has appeared to date that patients with these two related, yet distinctive, diseases have a similar HLA phenotype [5–7,9–10,12–14]. Recently, Howell and co-workers described a 4.0-kb RsaI RFLP using a DQ β-chain cDNA probe, PII β1 [15,21]. They detected this RFLP in 95% of patients with isolated GSE and in only 27% of normal HLA-DR3/DQw2 controls [15,21].

In order to determine if this 4.0-kb RsaI RFLP was also present in patients with DH, we have analyzed genomic DNA from 24 patients with DH using RsaI and the same DQ β-chain cDNA probe used by Howell et al [15]. We have found that 87% of our patients with DH have the 4.0-kb RFLP, which Howell et al found in 95% of their patients with isolated GSE. Since the major HLA association that is found in patients with DH is with HLA-DR3, -DQw2, we have examined 10 normal HLA-DR3, -DQw2 subjects for this RFLP and found that 7 of 10 (70%) HLA-DR3/DQw2 control subjects also had this 4.0-kb RsaI RFLP. Similar results have also been reported by Sacks and co-workers, who analyzed genomic DNA from patients with isolated GSE and normal HLA-DR3, -DQw2 control subjects and found no difference in the bands seen using RsaI and the cDNA DQ β-chain probe pII β-1 [22]. Our data document that the 4.0-kb RsaI RFLP found in patients with isolated GSE is also present in most patients with DH, further demonstrating the genetic similarity between patients with isolated GSE and patients with DH. However, much like the association of the HLA-A1 and HLA-B8 antigens in DH, the high frequency of this RFLP in normal HLA-DR3/DQw2 subjects suggests that this RFLP is not specific for either DH or isolated GSE, but rather may be a secondary association with either HLA-DR3, -DQw2, or other associated HLA antigens.

Although this 4.0-kb RsaI RFLP does not appear to be specific for either DH or isolated GSE, it could be strongly associated with the HLA-A1, -B8, -DR3, -DQw2 haplotype. One method of examining this question is to study families of patients with DH. If in the family studies the RFLP cosegregates with the clinical disease and the HLA-A1, -B8, -DR3, -DQw2 haplotype, it is more likely that

the RFLP and the disease are genetically linked. We have studied the families of 5 patients with DH for the presence of this RFLP. In three of the families, the RFLP could be assigned to a specific HLA haplotype (families 1, 2, and 3, Table II). In one instance, the 4.0-kb RFLP was present on the haplotype thought to be of primary importance in DH (i.e., the haplotype containing HLA-DR3 and -DQw2); however, in the other two families the RFLP did not segregate with this haplotype or with the clinical manifestations of the disease. In the remaining two families, the RFLP was also present in family members without the disease, including an unaffected sister (family 5), as well as in the patients with DH. These data document that this 4.0-kb RsaI RFLP is not predictive of either the clinical manifestations of DH or the HLA-A1, -B8, -DR3, -DQw2 haplotype, and suggests that the chromosomal region where this RFLP is found does not play a primary role in the pathogenesis of DH.

Howell and co-workers have localized this 4.0-kb RsaI RFLP to the region of the HLA-DP β-chain gene on chromosome 6 [15,21]. Recently, we have investigated the frequency of HLA-DP antigens, a third locus of expressed HLA class-II genes, in patients with DH [7]. Although both HLA-DPw1 and HLA-DPw3 were present more frequently in patients with DH (HLA-DPw1, 44%; HLA-DPw3, 32%) than in HLA-DR3 control subjects (HLA-DPw1, 26%; HLA-DPw3, 13%), this difference was not statistically significant when corrected for the number of alleles examined [7]. These data suggest that the positive association seen with HLA-DP antigens in patients with DH is, like the association with the class-I antigens HLA-A1 and -B8, most likely due to a linkage disequilibrium with the HLA class-II antigens, HLA-DR3 and -DQw2 [7]. We hypothesized at that time that the 4.0-kb RsaI RFLP found in isolated GSE may be associated with the HLA-DPw1 and/or HLA-DPw3 phenotypes. Recently, Kagnoff et al reported that patients with isolated GSE have an increased frequency of both HLA-DPw1 and HLA-DPw3, similar to that reported for patients with DH [23]. In addition, they reported that the presence of the 4.0-kb RsaI DP-β RFLP in patients with isolated GSE could be accounted for by the increased frequency of HLA-DPw1 and/or -DPw3 [23].

We have also investigated the relationship of this RFLP to HLA-DP phenotype in our patients and normal subjects who have been HLA-DP-typed (18 patients with DH, 9 nonaffected family members, and 5 non-HLA-DR3, -DQw2 normal subjects). Analysis of these 32 HLA-DP-typed subjects for the 4.0-kb RsaI RFLP revealed a significant association with HLA-DPw1 and/or HLA-DPw3 phenotype (two-tailed Fisher's exact test, $p = 0.00017$, uncorrected; $p = 0.0009$ corrected for five DP alleles tested for). Indeed, analysis of three cell lines homozygous for HLA-DPw3 and two cell lines homozygous for HLA-DPw1 revealed that even though none of these lines expressed either HLA-DR3 or -DQw2 all of the five cell lines were found to have the 4.0-kb RsaI RFLP. These data suggest that the 4.0-kb RsaI RFLP present in patients with DH is related to HLA-DP phenotype and not primarily associated with DH. Analysis of the family studies further supports this finding in that when the 4.0-kb RsaI RFLP could be assigned to a haplotype, it was associated with the HLA-DPw1 and/or -DPw3 containing haplotype.

We have found that 87% of patients with DH have a 4.0-kb RFLP using RsaI and the DQ β-chain cDNA pII β-1, similar to the frequency of this RFLP in isolated GSE. In addition, studies of HLA-DP-typed patients, families, and controls suggest that this 4.0-kb RsaI RFLP is not restricted to the haplotype thought to be important in the pathogenesis of DH (HLA-A1, -B8, -DR3, -DQw2), but rather its presence is related to the increased frequency of HLA-DPw1 and HLA-DPw3 in patients with DH. These data are in agreement with that reported by Kagnoff et al for patients with isolated GSE [23], and provide further documentation of the genetic similarity between patients with DH and with isolated GSE.

Recent evidence from a number of investigators has suggested that isolated GSE is primarily associated with HLA-DQ region genes [9,24,25]. Our data suggest that this HLA region is also of primary importance in the pathogenesis of DH. The lack of a statistically significant association with HLA-DPw1 or HLA-DPw3 antigens and DH, and the lack of tight genetic linkage between DH and the 4.0-kb RsaI DP β-chain region RFLP, suggest that the "DH haplotype" may not extend to the more centromeric HLA-DP region. These data also document the close similarity of the HLA class-II phenotype of patients with DH and isolated GSE, and suggest that the clinical characteristics which distinguish patients with DH from those with isolated GSE cannot yet be attributed to differences in HLA phenotype. It is possible, however, that molecular characterization of the HLA-DQ locus may reveal important differences between patients with DH and those with isolated GSE which may relate to the clinical differences observed.

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