Brief Definitive Report

AN HLA-D REGION RESTRICTION FRAGMENT LENGTH POLYMORPHISM ASSOCIATED WITH CELIAC DISEASE

By MARK D. HOWELL, RALEIGH K. AUSTIN, DERMOT KELLEHER, GERALD T. NEPOM,* AND MARTIN F. KAGNOFF

*From the Department of Medicine, University of California at San Diego School of Medicine, La Jolla, California 92093; and the *University of Washington School of Medicine, Seattle, Washington 98104*

Celiac disease is activated in genetically susceptible individuals by the ingestion of dietary gluten and similar proteins in other grains (reviewed in 1). The HLA class II specificity DQw2 (previously known as DC3 or MB2) is present, as assessed serologically, in 80–90% of individuals with clinically diagnosed celiac disease (2–4). However, ~25% of the normal population also has this serologic marker and ingests dietary gluten without the development of disease (3). This observation suggests that the HLA antigen associated with increased susceptibility to celiac disease may be a subspecificity of HLA-DQw2, or may be encoded by another HLA-D region locus that is in linkage disequilibrium with the DQ locus. To identify such a celiac disease–associated HLA-D region specificity, we examined the structure of class II HLA genes in celiac disease patients and control subjects using restriction fragment length polymorphism (RFLP) analysis (5). We now report a polymorphic 4.0 kb Rsa I fragment detected using a DQ β chain cDNA probe. This 4.0 kb fragment provides a more accurate means of identifying individuals at risk for the development of celiac disease than the serologic marker, HLA-DQw2.

Materials and Methods

Patient and Control Cell Lines. Patients for these studies were 20 unrelated Caucasians with clinically diagnosed celiac disease. Diagnosis was based on clinical evidence of malabsorption, a small bowel biopsy compatible with celiac disease, clinical and/or biopsy improvement on a gluten-free diet, and clinical and/or biopsy abnormalities upon rechallenge with a gluten-containing diet. Controls were 11 unrelated individuals without clinical evidence of celiac disease. Patients and controls in this study were HLA typed by a standard complement-dependent microcytotoxicity assay, and were all positive for at least one allele of DQw2. Lymphoblastoid B cell lines were initiated from donors by culturing 5×10^6 peripheral blood leukocytes with supernatant from the Epstein-Barr virus–producing cell line, 1437, in the presence of 2 μg/ml cyclosporin A. These B cell lines were maintained in RPMI 1640 culture medium supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% FCS.

DNA Purification. Nuclei from B cell lines were prepared by lysis of 10^8 cells in 20 ml of 10 mM Tris HCl (ph 8.0), 150 mM sodium chloride, 1 mM magnesium chloride, and 0.5% NP-40. After centrifugation at 750 *g*, the nuclear pellet was resuspended in 75 mM sodium chloride, 25 mM EDTA with gentle pipetting. SDS and proteinase K were added

This work was supported by grant AM35108 from the National Institutes of Health, Bethesda, MD.

to final concentrations of 0.5% and 200 μg/ml, respectively, and after 16 h at 37°C, solutions were extracted twice with phenol/chloroform/isopentyl alcohol (25:24:1), and once with chloroform/isopentyl alcohol (24:1). After the addition of one-half the volume of 7.5 M ammonium acetate, nucleic acids were precipitated with ethanol and dissolved in 10 mM Tris HCl (pH 8.0) with 1 mM EDTA (TE buffer).

RFLP Analysis. 15 μg of high molecular weight DNA from patient and control samples were digested for 16 h with 37.5 U of restriction endonuclease in accordance with manufacturer's specifications. Reactions were terminated by the addition of EDTA (10 mM final concentration) and ammonium acetate (2.5 M final concentration), and DNA was precipitated with ethanol. After centrifugation, DNA pellets were dried under vacuum and resuspended in TE. Restriction endonuclease–digested genomic DNA was electrophoresed in 0.8% agarose gels in Tris/acetate/EDTA (6). Gels were acid-pretreated (7) and blotted overnight onto nitrocellulose as described by Southern (8). Blots were baked 3 h at 80°C and hybridized essentially as described (7). Briefly, nitrocellulose was wet with 2× SSC (1× SSC is 150 mm sodium chloride, 15 mm sodium citrate), then prehybridized for 6 h at 42°C in 50% deionized formamide, 5× SSC, 5× Denhardt's additives, 50 mM sodium phosphate, and 250 μg/ml denatured salmon testes DNA. Subsequently, hybridization was conducted overnight at 42°C in 50% deionized formamide, 5× SSC, 5× Denhardt's additives, 20 mM sodium phosphate, 100 μg/ml denatured salmon testes DNA, 10% dextran sulfate, and 2×10^6 cpm/ml of probe nick-translated to $2–4 \times 10^8$ cpm/μg sp act. Blots were washed three times with 2× SSC, 0.1% SDS for 5 min at room temperature, twice with 0.1× SSC, 0.1% SDS for 30 min at 60°C and exposed to Kodak X-AR5 x-ray film with intensifying screens for 3 d.

Hybridization Probes. Probes used in these studies were molecularly cloned cDNAs encoding a DQ β chain (pII β-1)[9], a DR β chain (pII β-3)[10], and a DQ α chain (pII α-5)[11], all provided by Dr. D. Larhammar, University of Uppsala, Uppsala, Sweden.

Results

Patient and control DNA samples were digested with a panel of restriction endonucleases including Bam HI, Eco RI, Hind III, and Pst I, enzymes with hexanucleotide recognition sequences, and Rsa I, which cleaves at a tetranucleotide site. Digests were screened by Southern blot analysis using DQ and DR β chain cDNA probes.

The restriction endonuclease Rsa I revealed a disease-associated RFLP that was detectable by hybridization to the DQ β chain cDNA probe (Fig. 1). Of the 10 celiac disease patient DNA's shown in Fig. 1 (lanes *1–10*), 9 possessed a 4.0 kb fragment that was present in only one of the five control DNA samples in Fig. 1 (lanes *11–15*). This fragment was not observed in hybridizations that used DR β (pIIβ-3) or DQ α (pIIα-5) chain cDNA probes, indicating that it is not derived from these genes, but arises from the DQ β chain gene or one with a high degree of sequence homology. Table I lists, individually, the entire patient and control populations that we have screened to date. We have found 90% (18 of 20) of DQw2 celiac patients and 18% (2 of 11) of DQw2 controls that are positive for the 4.0 kb Rsa I fragment. Consequently, in DQw2 individuals, the relative risk (12) of developing celiac disease is 40-fold greater in the presence of this fragment than in its absence ($\chi^2 = 15.98$, $p \ll 0.001$).

Discussion

This study describes a means for distinguishing the HLA-D region haplotype associated with celiac disease from a serologically identical haplotype present in unaffected controls. The most common HLA-D region serologic haplotypes

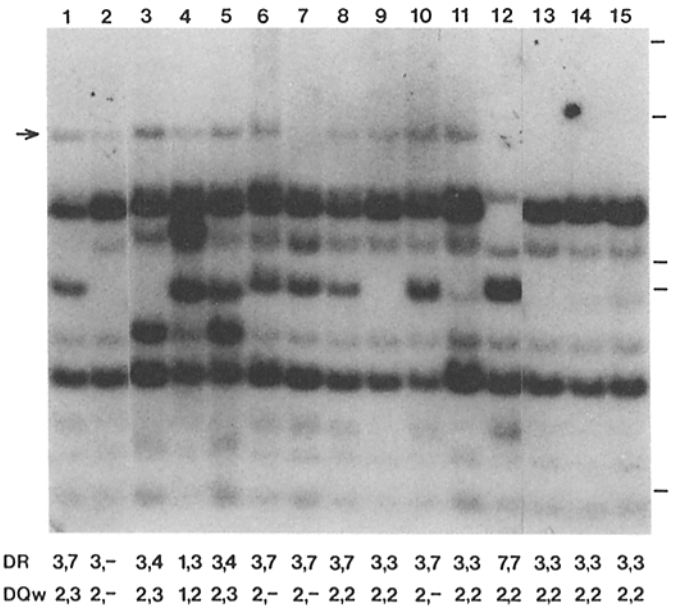

FIGURE 1. Rsa I–digested genomic DNA from celiac disease patients (lanes *1–10*) and controls (lanes *11–15*) hybridized to a 780-bp Pst I–Eco RI fragment of pII β-1 (DQ β probe). HLA-DR and -DQ types are indicated below each sample. Arrow shows position of 4.0-kb RFLP. Bars in the right hand margin denote mobilities of Hind III fragments of bacteriophage λ DNA (in descending order 6.6, 4.3, 2.2, 2.1, and 0.56 kb).

associated with celiac disease are HLA-DR3, -DQw2 and HLA-DR7, -DQw2. Each of the individuals in our patient and control populations was seropositive for one of these haplotypes, yet the 4.0 kb Rsa I fragment was predominantly associated with the HLA-D region genes of celiac disease patients. Given this strong association with disease, it is possible that the fragment is derived directly from a celiac disease susceptibility gene. Alternatively, it may be derived from an HLA-D region β chain gene that does not confer disease susceptibility but is in tight linkage disequilibrium with one that does. Regardless, the detection of this fragment provides a molecular genetic marker that can be used to identify the celiac disease haplotype, and may ultimately be used to locate the HLA-D region gene(s) predisposing for celiac disease.

All D region β chain genes share varying degrees of sequence homology, which permits crosshybridization in Southern blot analysis (5, 13–15). Therefore, detection of the 4.0 kb RFLP with a DQ β chain probe does not conclusively map this fragment to the DQ locus. Given the lack of hybridization to the DR probe, the 4.0 kb RFLP is probably not a DR β chain gene fragment. However, we cannot preclude the possibility that the 4.0 kb fragment is part of a β chain gene derived from a D region locus other than DQ or DR. The identity of the HLA-D region locus from which the fragment is derived is currently under study.

Finally, a gene fragment similar or identical to the 4.0 kb RFLP identified in our studies is also found in individuals with insulin-dependent diabetes mellitus (IDDM). Arnheim, et al. (16) have used RFLP analyses to study the HLA-D

TABLE I
Patient and Control Populations

Patient	DR	DQw	Presence of 4.0-kb RFLP
1	2, 3	1, 2	+
2	1, 3	1, 2	+
3	3, 7	2, 3	+
4	3, 7	2, —	+
5	3, 7	2, —	−
6	3, 4	2, 3	+
7	3, —	2, —	+
8	3, —	2, —	+
9	3, 7	2, 2	+
10	3, 3	2, 2	+
11	2, 7	1, 2	+
12	3, 6	1, 2	+
13	5, 7	2, 3	−
14	3, 5	2, 3	+
15	3, 6	1, 2	+
16	3, —	2, 3	+
17	3, —	2, —	+
18	3, 7	2, —	+
19	3, 2	1, 2	+
20	3, 4	2, 3	+
		Total:	18+, 2−
Control subject			
1	3, 3	2, 2	−
2	3, 3	2, 2	−
3	3, 3	2, 2	+
4	3, 3	2, 2	−
5	3, 3	2, 2	+
6	7, 7	2, 2	−
7	7, 7	2, 2	−
8	7, 7	2, 2	−
9	7, 7	2, 2	−
10	1, 7	1, 2	−
11	7, 9	2, —	−
		Total:	2+, 9−

region genes of IDDM patients. It is apparent from data in that report, although not specifically stated by the authors, that the 4.0 kb Rsa I fragment is present in the DNA of IDDM patients but not in that of controls. This is interesting, because an association between celiac disease and IDDM is well recognized (17). Serologically, a common denominator between celiac disease and IDDM is the increased frequency in both of HLA-DR3 (2). That the HLA-D region haplotypes associated with both diseases are similar at the DNA level suggests that the immunological mechanisms that mediate these disparate diseases share, to some degree, a common etiology.

Summary

This study is the first to describe a molecular marker that distinguishes the celiac disease HLA-D region haplotype from a serologically identical haplotype in unaffected controls. Using a DQ β chain cDNA probe and the restriction endonuclease Rsa I, we have detected a polymorphic 4.0 kb fragment which, in DQw2 individuals, is associated with a 40-fold increased relative risk of developing celiac disease. This finding should permit the identification of the celiac disease susceptibility gene(s) in the HLA-D region and facilitate a more precise

dissection of the molecular and immunogenetic mechanisms involved in the pathogenesis of that disease.

We thank Ms. K. O'Shaughnessy and Ms. D. Sagall for preparation of the manuscript.

Received for publication 21 April 1986.

References

1. Cole, S. G., and M. F. Kagnoff. 1985. Celiac disease. *Annu. Rev. Nutr.* 5:241.
2. Tiwari, J. L., and P. I. Terasaki. 1985. HLA and Disease Associations, Springer-Verlag, New York. 472.
3. Corazza, G. R., P. Tabacchi, M. Frisoni, C. Prati, and G. Gasbarrini. 1985. DR and non-DR Ia allotypes are associated with susceptibility to coeliac disease. *Gut.* 26:1210.
4. Tosi, R., D. Vismara, N. Tanigaki, G. B. Ferrara, F. Cicimarra, W. Buffolano, D. Follo, and S. Auricchio. 1983. Evidence that celiac disease is primarily associated with a DC locus allelic specificity. *Clin. Immunol. Immunopathol.* 28:395.
5. Cohen, D., P. Paul, I. LeGall, A. Marcadet, M.-P. Font, O. Cohen-Hagnenquer, B. Sayagh, H. Cann, J.-M. Lalouel, and J. Dausset. 1985. DNA polymorphism of HLA class I and class II regions. *Immunol. Rev.* 85:87.
6. Maniatis, J., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 545.
7. Wahl, C. M., M. Stern, and G. R. Stark. 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate. *Proc. Natl. Acad. Sci. USA.* 76:3683.
8. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503.
9. Larhammar, D., L. Schenning, K. Gustafsson, K. Wiman, L. Claesson, L. Rask, and P. A. Peterson. 1982. Complete amino acid sequence of an HLA-DR antigen-like β chain as predicted from the nucleotide sequence: Similarities with immunoglobulins and HLA-A, -B, and -C antigens. *Proc. Natl. Acad. Sci. USA.* 79:3687.
10. Gustafsson, K., K. Wiman, E. Emmoth, D. Larhammar, J. Bohme, J. Hyldig-Nielsen, H. Ronne, P. A. Peterson, and L. Rask. 1984. Mutations and selection in the generation of class II histocompatibility antigen polymorphism. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:1655.
11. Schenning, L., D. Larhammar, P. Bill, K. Winman, A-K. Jonsson, L. Rask, and P. A. Peterson. 1984. Both α and β chains of HLA-DC class II histocompatibility antigens display extensive polymorphism in their amino-terminal domains. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:447.
12. Svejgaard, A., C. Jersild, L. Staub-Nielsen, and W. F. Bodmer. 1974. HL-A antigens and disease: statistical and genetical considerations. *Tissue Antigens.* 4:95.
13. Andersson, M., J. Bohme, G. Andersson, E. Moller, E. Thorsby, L. Rask, and P. A. Peterson. 1984. Genomic-hybridization with class II transplantation antigen cDNA probes as a complementary technique in tissue typing. *Hum. Immunol.* 11:57.
14. Long, E. O., C. T. Wake, J. Gorski, and B. Mach. 1983. Complete sequence of an HLA-DR β chain deduced from a cDNA clone and identification of multiple non-allelic DR β chain genes. *EMBO (Eur. Mol. Biol. Organ.) J.* 2:389.
15. Gorski, J., P. Rollini, E. Long, and B. Mach. 1984. Molecular organization of the HLA-SB region of the human major histocompatibility complex and evidence for two SB β-chain genes. *Proc. Natl. Acad. Sci. USA.* 81:3934.
16. Arnheim, N., C. Strange, and H. Erlich. 1985. Use of pooled DNA samples to detect

linkage disequilibrium of polymorphic restriction fragments and human disease: studies of the HLA class II loci. *Proc. Natl. Acad. Sci. USA.* 82:6970.

17. Shanahan, F., R. McKenna, C. F. McCarthy, and M. I. Drury. 1982. Coeliac disease with diabetes mellitus: a study of 24 patients with HLA typing. *Q. J. Med.* 51:329.