THE HLA-DR4 FAMILY OF HAPLOTYPES CONSISTS OF A SERIES OF DISTINCT DR AND DS MOLECULES

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Ia antigens on lymphoid cells function as informational molecules that facilitate genetically regulated immune responses and cell-to-cell communication (1–4). In mice, two loci within the murine major histocompatibility complex (H-2), I-A and I-E, encode four polypeptides, with molecular weights of 34,000 daltons (the α chain) and 29,000 daltons, (the β chain) which associate into the heterodimers $A_\alpha A_\beta$ and $E_\alpha E_\beta$ (5). Over 40 murine haplotypes are known to exist at these loci, and further polymorphism probably exists among wild mice populations (6). Most of the functional attributes of Ia antigens are due to this genetic polymorphism: Strains that differ in their Ia structure, predominantly β chain variations, differ in their capacity for some immune responses and are mutually reactive in MLR.[1]

The analogous genetic system in man, HLA, appears to be somewhat more complicated than the murine counterpart. The HLA-D locus was originally defined as the genetic region causing stimulation in MLR (MLR-S) (7). Human Ia antigens map to the HLA-D region (8). These appear to be encoded by at least three distinct loci, DR, DS (or DC), and SB, each with its distinct α and β chains. The DRα chain is structurally analogous to the murine I-Eα chain, and the DC/DSα chain is structurally analogous to the murine I-Aα chain (9–11). The relationship between HLA-D or MLR-S, and the human "Ia-like" antigens remains largely unknown. Within the DR4 haplotype, analysis by MLR has led to the description of several distinct HLA-D antigens, including Dw4, Dw10, LD″40″, LD″DYT″, LD″KT2″, and LD″TAS″ (12, 13).

Among particular HLA specificities associated with prevalent diseases, HLA-DR4 has attracted much interest. This antigen is found to be more frequent in patients with adult rheumatoid arthritis, insulin-dependent diabetes, and some forms of juvenile rheumatoid arthritis, than in the general population. The strength of these associations varies in different racial groups, raising the question

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[1] *Abbreviations used in this paper:* EBV, Epstein-Barr virus; HCL, homozygous B-lymphoblastoid cell line; HTC, homozygous typing cells; MLR, mixed lymphocyte reaction; PAGE, polyacrylamide gel electrophoresis; SaCl, formalin-fixed heat killed Cowan 1 strain staphylococcus aureus.

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of heterogeneity within the clinical spectrum of disease or within the associated genetic factors. Groner et al. (14) have utilized two-dimensional polyacrylamide gel electrophoresis (PAGE) to demonstrate molecular heterogeneity within the HLA-DR4 haplotype (14). We have recently described a similar study in which six distinct DR β chain patterns were identified among 17 B-lymphoblastoid cell lines derived from donors of DR4-associated HLA-D homozygous typing cells (HTC) analyzed by two-dimensional PAGE (12). When DR β chains representative of different HLA-D clusters were compared, the patterns correlated precisely with the HLA-D phenotype of the HTC donor. Thus, we suggested in agreement with Groner et al. (14) that HLA-D specificities could be accounted for by structural variations in the DR β chain.

Phenotypic variation as defined by MLR, however, is likely to represent the composite expression of the entire repertoire of human "Ia-like" antigens, not just the products of the DR β locus. Indeed, it is quite surprising that analysis of a single component of the "D-region," the DR β chains, showed such striking correlation with the MLR-defined HLA-D antigen clusters (12, 14). We have now investigated each of the DR4 homozygous cell lines (HCL) in more detail. Neuraminidase treatment of immunoprecipitated DR antigens, followed by two-dimensional gel electrophoresis, clarifies the extent to which glycosylation contributes to DR β chain variability. We also report here the existence of structural heterogeneity within the DR4 haplotype for the products of a second human "Ia-like" locus HLA-DS (or DC). Based on the observed differences for DR and DS β chains five distinct DR4 haplotypes are defined. This analysis may provide some insight into the diversity of genetic factors associated with disease.

Materials and Methods

Homozygous B-Lymphoblastoid Cell Lines. Lymphoblastoid cell lines from DR4-associated HLA-D HTC were established as previously described (12, 15). Briefly, $1-2 \times 10^6$ B cells/ml from donors whose lymphocytes have been used as HTC were mixed with an equal volume of culture supernatant from the EBV secreting line B95-8, plated into microwell tissue culture plates, and expanded within 15–60 d. The cell lines used for this study were: FS and EM (Dw10); WALK, HA, PRIESS, and ER (Dw4); LS40 and BIN 40 (LD″40″); KT3 and HAS-15 (LD″DYT″); JHa and SST (LD″DB3″); KT2 and KT13 (LD″KT2″); and TAS (LD″TAS″). All cell lines have been described in detail elsewhere (12). The HLA-DR phenotype was confirmed as DR4 for each line by standard HLA-DR typing procedures (16, 17).

Monoclonal Anti-Ia Antibodies. Antibody P4.1, a murine monoclonal IgG_{2a} antibody specific for HLA-DR molecules ("monomorphic") has been described in detail elsewhere (12). Antibody SG157 is also specific for a determinant on DR (I-E–like) molecules as shown by amino acid sequence analysis (18). In contrast, antisera and monoclonal antibodies recognizing "I-A–like" antigens distinct from DR products have been recently described (19). Monoclonal antibody SG465 recognizes a determinant on DS ("I-A–like") antigens (20).

Radiolabeling and Immunoprecipitation. Cell labeling with ^{125}I was performed as described previously (12). Briefly, 4×10^7 cells in 0.5 ml phosphate-buffered saline with 5 mM glucose, 50 μg glucose oxidase, and 0.7 U lactoperoxidase (Calbiochem-Behring Corp., La Jolla, CA) were incubated with 2 mCi Na ^{125}I (Amersham, Arlington Heights, IL) for 20 min at room temperature. Cells were lysed as previously described (12) and lysates recovered after pre-clearing with washed heat-killed Cowan 1 strain *Staphylococcus aureus* (SaC1, BRL, Bethesda, MD). Lysate was incubated overnight with the appropriate monoclonal antibody, precipitated with SaC1, and washed repetitively.

For sequential immunoprecipitations, lysates were precipitated with the first antibody and the resultant supernatants were then re-precipitated with a second antibody.

Neuraminidase Treatment of Cells. Neuraminidase treatment was carried out using the procedure of Shackelford et al. (21). Briefly, 150 μl of radiolabeled lysate prepared as above was treated with a total of 150 μl of neuraminidase (Calbiochem-Behring Corp.) over the course of 5 h at 37°C and then immunoprecipitated as described above.

Two-dimensional Gel Electrophoresis. Samples were electrophoresed using a modification of the O'Farrell system for nonequilibrium electrofocusing (22), followed by 10% polyacrylamide slab gel electrophoresis, as described previously (12, 23).

Additive gels were performed by mixing lysate samples of equivalent ^{125}I cpm immediately before loading on the first-dimension gel apparatus.

Direct comparisons between separate samples were made only on gels that were simultaneously prepared and electrophoresed side-by-side on the same apparatus.

Results

Identification of Distinct DR4-Associated DR β Chain (I-E–like) Variants Corresponding to HLA-D. Two-dimensional gel electrophoresis of immunoprecipitated β chains from homozygous cell lines HA (DR4, Dw4) and EM (DR4, Dw10) were compared (Fig. 1). Precipitations performed using antibody P4.1, an anti-DR "monomorphic" monoclonal antibody, showed marked differences in electrophoretic mobility between Dw4 and Dw10 DR β chains. Most, if not all, of the charge heterogeneity within each immunoprecipitated glycoprotein appeared to be due to post-translational sialylation of the oligosaccharide component of the β chain, since treatment with neuraminidase greatly simplified the electrophoretic pattern. In each case, acidic spots disappeared while the most basic spots increased in intensity.

Similar electrophoretic profiles of immunoprecipitated β chains following neuraminidase treatment were obtained for all 15 DR4-associated HLA-D homozygous cell lines listed in Table I. Gels from some of these are shown in Fig. 2, along with "additive" gels, in which immunoprecipitates from two cell lines were mixed and co-electrophoresed in order to clarify the relationship between similar,

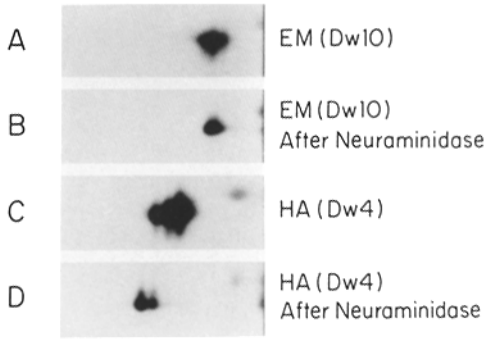

FIGURE 1. Two-dimensional polyacrylamide gel electrophoresis of immunoprecipitated DR "Ia-like" molecules from HCL EM and HA. Autoradiograms shown display the "Ia-like" β chain, oriented with anode (pH 5) on the right, cathode (pH 8.5) on the left, and with higher M_r at the top and lower M_r at the bottom. Panels *A* and *C* show the precipitates from cell lines EM and HA without treatment with neuraminidase, and panels *B* and *D* show the precipitates after neuraminidase digestion. All gels were electrophoresed simultaneously on the same apparatus to facilitate direct comparisons.

TABLE I

Classification of DR and DS β Chains Isolated from HLA-DR4–associated HLA-D Homozygous Typing Cells

HLA-D	HCL	Electrophoretic variants	
		DR	DS
Dw10	FS, EM	$DR4_{\beta 1}$	$DS4_{\beta 1}$
Dw4	WALK, HA, PRIESS, ER	$DR4_{\beta 2a}$	$DS4_{\beta 2}$
LD″40″	BIN40, LS40	$DR4_{\beta 2b}$	$DS4_{\beta 2}$
LD″DYT″	KT3, HAS-15	$DR4_{\beta 3}$	$DS4_{\beta 2}$
LD″TAS″	TAS	$DR4_{\beta 4}$	$DS4_{\beta 3}$
LD″DB3″	JHa, SST	$DR4_{\beta 5}$	$DS4_{\beta 3}$
LD″KT2″	KT2, KT13	$DR4_{\beta 5}$	$DS4_{\beta 3}$

The sources and derivation of these HCL have been described elsewhere (12). DR β chains were isolated with antibody P4.1 and DS β chains were isolated with antibody SG465. Types DR2a and DR2b are electrophoretically distinct before, but not after, neuraminidase.

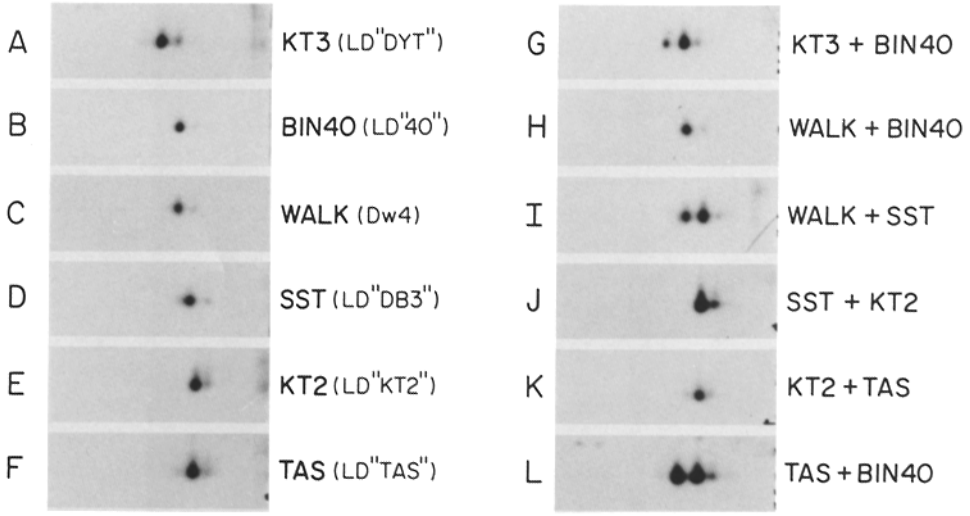

FIGURE 2. Two-dimensional polyacrylamide gel patterns of neuraminidase-treated immunoprecipitates from DR4 HCL from different HLA-D clusters. Shown in panels *A–F* are profiles of the β chain immunoprecipitates obtained from one representative cell line of each of the known DR4-associated HLA-D clusters, except for the Dw10 cluster (The Dw10 β chain pattern is shown in Fig. 1). Shown in panels *G–L* are profiles from "mixing" gels in which two separate immunoprecipitates were combined before electrophoresis in order to discern subtle differences in electrophoretic mobility between samples. Multiple samples from each HLA-D cluster were analyzed, and in the cases where the observed gel patterns from HCL within the same cluster were identical, only one representative gel is shown.

but nonoverlapping, spots. All gels shown were electrophoresed simultaneously on the same apparatus, to minimize inter-sample variation. The examples shown in Fig. 2 illustrate the differences found between DR β chain polypeptides from different HCL. Thus, neuraminidase-treated DR antigens from lines KT3 (LD″DYT″) and BIN 40 (LD″40″) both gave a pattern consisting of a dominant basic spot with a faint, slightly more acidic spot (possibly representing incomplete

sialic acid removal). When aliquots of the two precipitates were mixed and co-electrophoresed (panel *G*) three distinct spots were seen, indicating that the KT3 major spot is more basic than the BIN 40 major spot. A similar situation was observed when the gel patterns for cell lines SST (LD″DB3″) and WALK (Dw4) were compared. In this case, each neuraminidase-treated precipitate contained a major basic spot and a faint, slightly more acidic spot, but the "combined" gel indicates that the major spot from WALK was more basic than the SST major spot (panel *I*). An additive gel comparing WALK and BIN 40 in panel *H* indicated that these two profiles were identical, since the same pattern was observed in the "combined" gel as in the gels for each cell line studied alone. Additional comparisons of cell lines TAS (LD″TAS″) and KT2 (LD″KT2″) with each other and with cell lines BIN 40 (LD″40″) and SST (LD″DB3″) are shown in panels *J–L* and indicate that the SST and KT2 profiles are identical. The profile for TAS was similar to that of SST and KT2, but had an additional faint basic spot that migrated slightly more anodally than the major BIN 40 spot. In additional immunoprecipitates (not shown), cell line EM (Dw10) was identical to FS (Dw10), HA (Dw4) and LS40 (LD″40″) were identical to WALK (Dw4), and JHa (LD″DB3″) and KT13 (LD″KT2″) were identical to KT2 (LD″KT2″).

In all, we observed five distinct gel patterns for the DR4 β chain following neuraminidase treatment. The pattern from cell lines FS and EM was markedly different from all the others, suggesting that the Dw10 cluster expresses a DR β chain only distantly related to the DR β chain of the other DR4-associated HLA-D clusters. The differences between the other DR4 HCL, although not as dramatic, were still distinct, and correlated in a consistent way with the MLR-defined D antigen of each HCL. DR β chains from HCL of the LD″DYT″ and LD″TAS″ clusters had unique, consistent profiles; those from HCL of the LD″DB3″ and LD″KT2″ clusters were identical to each other, but distinct from all the other DR4 HCL; those from HCL of the Dw4 and LD″40″ clusters were identical to each other, but distinct from all the others. Neuraminidase-treated DR chains from these latter four clusters (LD″DYT″, LD″TAS″, LD″KT2″, and Dw4) gave gel patterns that differed from each other in only one or two charge units per molecule. Such small differences probably reflect minor amino acid variations, and suggest close evolutionary homology. Since these findings correlated in a consistent way with MLR reactivity, the differences seen for the DR β chains may well be sufficient to explain the polymorphism based on HLA-D typing.

No difference was found between the DR β chain from LD″DB3″ and LD″KT2″. This is consistent with our previous report that gel patterns from these cells before neuraminidase treatment were identical (12). These two HLA-D clusters were originally defined in different ethnic populations, but more recent data from a direct comparative study of the two has suggested that ″DB3″ and ″KT2″ may in fact be identical (13).

The Dw4 and LD″40″ comparison is more intriguing. We have previously reported that the DR β chain profiles from these two D clusters differ in electrophoretic mobility (12). Before neuraminidase treatment, the three β chain spots present in the LD″40″ profile were identical to three of the β chain spots in the Dw4 profile, but two additional spots were seen in the Dw4 profile.

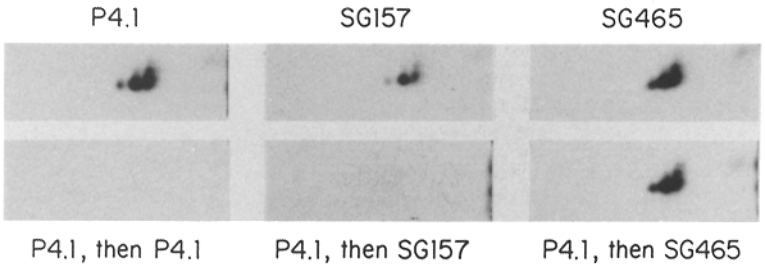

FIGURE 3. Sequential immunoprecipitation and two-dimensional gel electrophoresis of DR4 "Ia-like" antigens using monoclonal antibodies P4.1 and SG465. Cell line HA(DR4,Dw4) was radiolabeled and a lysate prepared as before. Immunoprecipitates using either antibodies P4.1 (anti-DR), SG157 (anti-DR), or SG465 (anti-DC/DS) were then used to obtain the gel patterns shown in the top panels. Lysates that had been precipitated with P4.1, and therefore "cleared" of DR molecules, were then re-precipitated with each of the same three antibodies. The resultant gel patterns from these sequential immunoprecipitations are shown in the bottom three panels.

Following neuraminidase treatment the DR β chain profiles of the Dw4 and LD″40″ antigens were indistinguishable. Thus, the primary polypeptides of these two DR chains appear to be identical and the variation observed between Dw4 and LD40 appears to be due to posttranslational changes in the number of sialic acid residues.

Identification of Distinct DR4-Associated DS β Chain (I-A–like) Variants. The relative contributions of different human "Ia-like" antigens to immune function is not known. In the mouse, strains that are identical at one locus, such as I-E, but different at the other, I-A, are mutually reactive in MLR. Assuming that a parallel situation exists in man, the composite array of all expressed "Ia-like" antigens, rather than solely the products of the DR (I-E like) locus, is likely to cause stimulation in MLR. To investigate this we analyzed electrophoretic patterns of β chains immunoprecipitated from HCL HA (DR4,Dw4) using antibodies P4.1, SG465, and SG157 (Fig. 3). Electrophoretic profiles of the immunoprecipitated "Ia-like" antigens (upper panels) were very similar for each of the three antibodies, but important differences were revealed by a sequential immunoprecipitation procedure: Cell lysates containing "Ia-like" antigens were first immunoprecipitated with antibody P4.1 to remove DR molecules, and were then re-precipitated with either antibody P4.1, SG157, or SG465. As shown in the lower panels of Fig. 3, this procedure removed, as expected, DR antigens reactive with antibodies P4.1 or SG157, but left the "Ia-like" DS molecules precipitated by antibody SG465. This result is consistent with the findings recently published by Goyert et al. (20).

Immunoprecipitates from HCL representing each of the DR4-associated HLA-D antigen clusters were then analyzed using antibody SG465. Radiolabeled lysates from each HCL were immunoprecipitated first with antibody P4.1 to remove DR molecules, and then with antibody SG465, to identify DS molecules. Several electrophoretic DS β chain variants were identified among the cell lines analyzed. These are shown in Fig. 4. The profiles of HCL FS and EM (Dw10) were identical with each other, but were different from the profiles obtained from the other DR4 HCL. DS β chain profiles from HCL TAS (LD″TAS″), SST

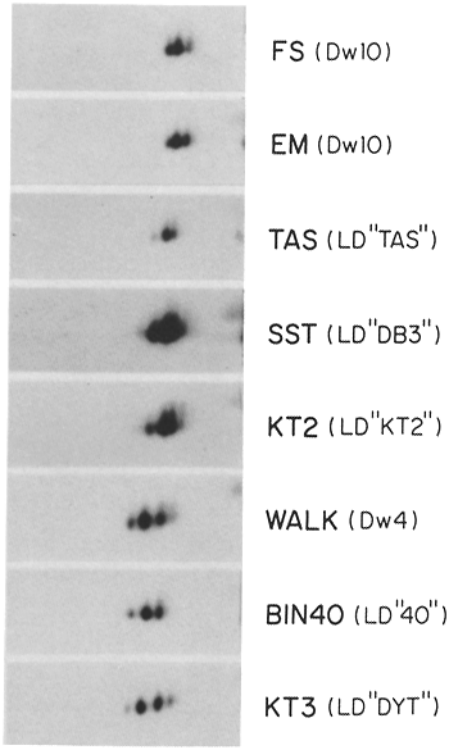

FIGURE 4. Two-dimensional gel electrophoresis of DC/DS "Ia-like" non-DR molecules from DR4 HCL. Radiolabeled lysates from each of the DR4 HCL shown were prepared as before, and first precipitated with antibody P4.1 to remove DR "Ia-like molecules". These same lysates were then immunoprecipitated using antibody SG465, an anti-DC/DS monoclonal antibody, followed by electrophoresis and autoradiography. All gel profiles are oriented the same as for the DR gels in previous figures; all gels were simultaneously electrophoresed on the same apparatus to facilitate comparisons.

(LD″DB3″), and KT2 (LD″KT2″) were identical, and migrated more cathodally than the FS and EM molecules. The DS β chains obtained from WALK (Dw4), BIN40 (LD″40″), and KT3 (LD″DYT″), were identical to each other. The DS β chains from this latter group were more basic than those of the other groups and appeared to correspond to the previously published description of DR4-associated ″DS″ molecules obtained from cell line GM3103 (″WT51″) (20).

Discussion

We demonstrate, using two-dimensional PAGE, distinct polymorphic β chains corresponding to both DR and DS gene products within DR4 homozygous cell lines. Five distinct polypeptides corresponding to DR4 products and three distinct polypeptides corresponding to DC/DS4 products were identified. Thus, there are at least five different haplotypes associated with the HLA-DR4 specificity, as defined by standard typing using alloantisera. Since the observed DR β chain gel patterns correlate with the HLA-D specificity identified by cluster analysis of MLR, it is apparent that the DR4 specificity is supertypic to "Ia-like" molecules of several D clusters. In other words, HLA-DR4 is a public serologic specificity

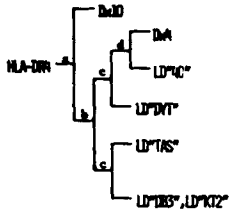

FIGURE 5. A "family tree" of DR4-associated HCL.

shared by different "Ia-like" molecules that are present on at least five different HLA haplotypes.

Comparison of gel patterns suggests several other observations: First, within the DR4 haplotype, there appears to be less heterogeneity for DS products, as compared with DR, since we can identify five different DR β chains and only three DS β chain patterns among the HCL studied. Second, the DS β chain patterns do not consistently correlate with the known intra-DR4 differences that account for reactivity in MLR. Thus, variation for DR β but not DS β appears to be a more consistent marker for HLA-D. Third, the similarity between DS β chains from different DR4 HCL suggests a close relationship among certain cells with different D haplotypes and different DR molecules. Thus, the DS β chains isolated from WALK, BIN40, and KT3 were indistinguishable, suggesting that the LD″DYT″ cluster may be closer structurally to the Dw4 and LD″40″ clusters than to other DR4 clusters. Similarly, the DS β chain expressed by TAS is identical to that on SST and KT2, and suggests that LD″TAS″ may be evolutionarily closer to the LD″KT2″ and LD″DB3″ clusters than to Dw4, Dw10, or LD″40″.

In several respects, the data from the DS immunoprecipitations reinforce our previous conclusions based on the neuraminidase-treated DR immunoprecipitates described above. That is, the Dw10 HCL FS and EM are markedly different from all other DR4 HCL for both DR β and DS β, indicating that the Dw10 cluster has little in common with other DR4 clusters. Also, the Dw4 HCL WALK and the LD″40″ HCL BIN40 have identical DS β chains, indicating a close relationship consistent with their similarity following neuraminidase treatment of DR molecules. Finally, the DS β chains of HCL LD″DB3″ and LD″KT2″ were indistinguishable, as were their DR β molecules.

From these data, it is clear that DR4 is not a single haplotype. Distinct and consistent structural variations exist for the β chains of two loci, DR and DS. It is currently not known whether the serologically defined DR4 specificity is carried by molecules encoded in the DR locus, the DS locus, both or neither. A composite diagram summarizing the relatedness of the different DR4-associated HLA-D clusters is illustrated in Fig. 5. The relationships implied by the "family tree" are meant to represent levels of structural similarity and not genetic map distance or a precise evolutionary scale. Branch point *a* separates Dw10 from the other DR4-associated D-clusters based on the identification of unique DR β and DS β chains ($DR4_{\beta 1}$, $DS4_{\beta 1}$) on the Dw10 HCL. Branch point *b* represents a separation of the remaining DR4 haplotypes into one of two distinct DS groups. Within each of these two, further subdivision is indicated at branch point *c*,

representing further structural variants of DR β following neuraminidase treatment, and therefore probably reflecting allelic variations in amino acid composition of related gene products. Branch point *d* notes our observation that Dw4 and "LD40" appear to differ only in their posttranslational glycosylation and thus may determine another distinct haplotype. These data are also summarized in Table I.

In all probability, the finding of considerable heterogeneity within a single DR haplotype is not unique to DR4. Our recent understanding of the molecular complexities of human "Ia-like" genes and polypeptides requires a reassessment of existing assumptions. The haplotypes DR1 through DRw10 were identified by association analysis of the reactivity of selected alloantisera. It has been apparent for some time, however, that more than 10 human D/DR haplotypes exist, since multiple distinct D antigens have been found to be associated not only with DR4 but also with DR2, DRw6, DR7, and DRw8, indicating that HLA-DR is supertypic to HLA-D (13, 24–36). Gel analysis of "Ia-like" molecules from the DR2-associated HLA-D antigens Dw2 and Dw12 (T. Sasazuki, personal communication) has reportedly shown some variations similar to those described for DR4 (12, 14).

The demonstration of heterogeneity within a group of haplotypes serologically defined as equivalent, e.g., DR4, provides a significant insight into the complexity of human "Ia-like" antigens. Ia structural polymorphism contributes a critical determinant controlling the recognition of antigen by cooperating lymphoid cells. Thus, diversity of Ia as shown here not only accounts for patterns of T cell reactivity in MLR, but may also account for some of the polymorphic features fundamental to "Ia-regulated" immune responses and to D/DR-associated disease susceptibility. Indeed, it is possible that some disease-associated genetic markers will be found on unique products of individual loci, not as a public serologic specificity defined by conventional typing reagents.

Summary

Among DR4-associated HLA-D antigens, distinct and consistent structural variations were found for the products of two human "Ia-like" loci, DR and DS. Analysis of neuraminidase-treated immunoprecipitated DR molecules from 15 HLA-DR4–associated HLA-D homozygous B-lymphoblastoid cell lines by two-dimensional polyacrylamide gel electrophoresis identified five distinct DR β chains. In addition, gel analysis of immunoprecipitated DS molecules identified three distinct DS β chains. Altogether, five distinct DR4 haplotypes were defined according to the observed structural diversity of the DR and DS β chains. These gene products presumably contribute the dominant polymorphisms recognized by T cells in mixed lymphocyte reaction (MLR). Thus, these studies indicate that the serologic specificity known as HLA-DR4 is not a single haplotype, but a determinant present on products of individual loci arrayed into distinctly different haplotypes. These findings suggest that distinct products of individual loci, rather than conventional HLA specificities defined by alloimmune sera, may represent the genetic markers relevant to HLA-D/DR associated diseases.

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